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(54) **Title:** NOVEL HUMAN CYTOMEGALOVIRUS DNA CONSTRUCTS AND USES THEREFOR

(57) **Abstract**

Novel DNA molecules for *in vitro* and *in vivo* expression of HCMV gB, gB transmembrane-deleted derivatives, pp65, pp150, and IE-exon-4 proteins are described. Preferably, the molecules are plasmids. Also described are methods of using these DNA molecules to induce immune responses to HCMV, and the use of a plasmid of the invention to prime immune responses to HCMV vaccines.

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NOVEL HUMAN CYTOMEGALOVIRUS DNA CONSTRUCTS AND USES THEREFOR

Field of the Invention

This invention relates generally to compositions useful in preventing and treating human cytomegalovirus infection.

Background of the Invention

Cytomegalovirus (CMV) is one of a group of highly host specific herpes viruses that produce unique large cells bearing intranuclear inclusions. The envelope of the human cytomegalovirus (HCMV) is characterized by a major glycoprotein complex termed gB or gCI, which was previously referred to as gA.

Infection with HCMV is common and usually asymptomatic. However, the incidence and spectrum of disease in newborns and immunocompromised hosts establishes this virus as an important human pathogen. HCMV has also been suggested to be an important co-factor in the development of atherosclerosis and restenosis after angioplastic surgery.

Several HCMV vaccines have been developed or are in the process of development. Vaccines based on live attenuated strains of HCMV have been described. [See, e.g., S. A. Plotkin et al, Lancet, 1:528-30 (1984); S. A. Plotkin et al, J. Infect. Dis., 134:470-75 (1976); S. A. Plotkin et al, "Prevention of Cytomegalovirus Disease by Towne Strain Live Attenuated Vaccine", in Birth Defects, Original Article Series, 20(1):271-287 (1984); J. P. Glazer et al, Ann. Intern. Med., 91:676-83 (1979); and U. S. Patent 3,959,466.] A proposed HCMV vaccine using a recombinant vaccinia virus expressing HCMV glycoprotein B has also been described. [See, e.g., Cranage, M. P. et al, EMBO J., 5:3057-3063 (1986).] However, vaccinia vaccines are considered possible causes of encephalitis. Other recombinant HCMV vaccines have been described.

See, e.g., G. S. Marshall et al, J. Infect. Dis., 162:1177-1181 (1990); K. Berencsi et al, J. Gen. Virol., 74:2507-2512 (1993), which describe adenovirus-HCMV recombinants.

There remains a need in the art for additional compositions useful in preventing CMV infection by enhancing immune responses to HCMV vaccines and generating neutralizing antibody and/or cellular responses to CMV in the human immune system.

Summary of the Invention

The present invention provides a series of DNA molecules expressing human cytomegalovirus (HCMV) genome fragments, which are particularly useful in inducing HCMV-specific immune responses.

Thus, in one aspect, the invention provides a DNA molecule which is non-replicating in mammals and which comprises at least one human cytomegalovirus antigen which is operably linked to regulatory sequences which express the antigen in the mammal. Advantageously, the antigen elicits an immune response in said mammal. In one preferred embodiment, the DNA molecule is a plasmid.

In another aspect, the invention provides a plasmid, pTet-gB, containing the portion of the HCMV genome (UL55) encoding gB. This plasmid further contains a tetracycline regulatable HCMV-immediate early promoter, which is useful in controlling expression of gB. Another plasmid of the invention encoding the full-length gB subunit protein is a pΔRC-gB plasmid.

Yet another plasmid of the invention, pΔRC-gB_{680}, contains the portion of the HCMV genome encoding the N-terminal 680 amino acids of the gB protein (gB_{1-680}).

The pΔRC-pp65 plasmid of the invention contains the portion of the HCMV genome (UL83) encoding the HCMV pp65 tegument protein. The pΔRC-pp150 plasmid contains the

portion of the HCMV genome (UL32) encoding the HCMV pp150 tegument protein.

The pΔRC-exon-4 contains the portion of the HCMV genome (truncated UL123) encoding HCMV immediate-early (IE) exon-4.

In yet another aspect, the present invention provides an immunogenic composition of the invention comprising at least one of the DNA molecules of the invention and a carrier.

In still another aspect, the present invention provides a method of inducing HCMV-specific immune responses in an animal by administering to the animal an effective amount of an immunogenic composition of the invention. Preferably, this composition contains pΔRC-gB_{680}, pTet-gB and/or pΔRC-pp65.

In yet a further aspect, the present invention provides a method of priming immune responses to a selected human cytomegalovirus immunogenic composition by administering an immunogenic composition of the invention prior to administration of the second immunogenic or vaccine composition.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 illustrates the construction of the pTet-gB plasmid.

Fig. 2 is a graph illustrating the results of pp65-specific CTL responses in BALB/c mice immunized with pΔRC-pp65. The circle represents VacWR-pp65-infected MC57 (MHC-mismatched) target cells; the diamond represents WT-Vac-infected P-815 cells; and the square represents VacWR-pp65-infected P-815 (MHC-mismatched) target cells.

Fig. 3A-3E provides the full-length DNA and amino acid sequences [SEQ ID NO:1 and 2] of a human cytomegalovirus virus gB gene.

Fig. 4A - B provide the full-length DNA and amino acid sequences [SEQ ID NO:3 and 4] of a human cytomegalovirus immediate-early exon-4.

Fig. 5 provides the full-length DNA and amino acid sequences of a human cytomegalovirus phosphoprotein (pp) 65 gene Towne strain on the top line [SEQ ID NO: 5 and 6], and, on the bottom line, the sequence of the pp65-AD169 strain where it differs from the Towne strain [SEQ ID NO: 7 and 8].

Fig. 6A - B provide the full-length DNA and amino acid sequences [SEQ ID NO: 9 and 10] of a human cytomegalovirus phosphoprotein (pp) 150 gene, AD169 strain.

Fig. 7A provides a circular map of the eukaryotic expression vector pCB11.

Fig. 7B provides a circular map of pCBgB.

Fig. 7C provides a circular map of pCBgBΔtm.

Fig. 8 provides a schematic representation of the gB protein (top line) and of its homolog which is deleted of the transmembrane domain (bottom line).

Fig. 9 is a graph illustrating the anti-gB titers in sera of BALB/c mice immunized with plasmids pCBgB and pCBgBΔtm intramuscularly (IM) and intradermally (ID).

Detailed Description of the Invention

The present invention provides DNA molecules useful for *in vitro* and *in vivo* expression of antigenic fragments of the HCMV genome. Particularly desirable antigens include full-length and transmembrane-deleted fragments of gB such as gB_{1-680}, pp65, pp150, and IE-exon-4. Preferably, the DNA molecules of the invention are plasmids. The inventors have found that these DNA

molecules induce HCMV-specific immune responses, including ELISA and neutralizing antibodies and cytotoxic T lymphocytes (CTL), and are further useful in priming immune responses to subsequently administered HCMV immunogens and vaccines.

Thus, in one embodiment, the present invention provides a DNA molecule containing at least one HCMV antigen under the control of regulatory sequences which express the antigen in vivo or in vitro. Desirably, the DNA molecule is incapable of replicating in mammals. In a particularly desirable aspect of this embodiment, the DNA molecule is a plasmid.

As defined herein, an HCMV antigen includes a portion of the HCMV genome or a protein or peptide encoded thereby which induces an immune response in a mammal. Desirably, the immune response induced is HCMV-specific and protective. However, non-protective immune responses are also useful according to the invention, e.g., for priming immune responses. Currently, preferred HCMV antigens include full-length gB, a fragment or derivative of gB which lacks at least the transmembrane domain, pp65, pp150, and the immediate-early exon-4. Other suitable antigens may be readily selected by one of skill in the art.

The exemplary DNA molecules of invention, described herein, have been constructed using gene fragments derived from the Towne strain of HCMV. The Towne strain of HCMV, is particularly desirable because it is attenuated and has a broad antigenic spectrum. This strain is described in J. Virol., 11 (6): 991 (1973) and is available from the ATCC under accession number VR-977. The Ad169 strain is also available from the ATCC, under accession number VR-538. However, other strains of CMV useful in the practice of this invention may be obtained

from depositories like the ATCC or from other institutes or universities, or from commercial sources.

Thus, the CMV gene fragment encoding the desired protein (e.g., gB, pp65, pp150) or protein fragment (e.g., gB_{1-680} or IE-exon-4) may be isolated from known HCMV strains. See, e.g., Mach et al, J. Gen. Virol., 67:1461-1467 (1986); Cranage, M. P. et al, EMBO J., 5:3057-3063 (1986); and Spaete et al, Virol., 167:207-225 (1987), which provide isolation techniques. For example, using a known HCMV sequence, the desired HCMV gene or gene fragment [e.g., pp65 (UL83)] is PCR amplified, isolated, and inserted into the plasmid vector or other DNA molecule of the invention using known techniques. Alternatively, the desired CMV sequences can be chemically synthesized by conventional methods known to one of skill in the art, purchased from commercial sources, or derived from CMV strains isolated using known techniques.

If desired, the DNA molecules of the invention may contain multiple copies of the HCMV gene or gene fragment. Alternatively, the recombinant plasmid may contain more than one HCMV gene/gene fragment, so that the plasmid may express two or more HCMV proteins. For example, as shown herein, the presence of both gB- and pp65-specific ELISA antibodies and pp65-specific CTL in the mice inoculated with pTet-gB and pΔRC-pp65 in a mixture indicates that gB and pp65 do not mutually block antigen presentation or B and T cell stimulation when expressed in the same cells or in close proximity. Thus, gB (or gB_{680}) and pp65 proteins are particularly well suited for incorporation into a plasmid which expressed both protein (termed herein a chimeric vector). Thus, one particularly desirable embodiment of the present invention provides a DNA molecule containing the gB and the pp65 antigens. In another particularly desirable

embodiment, the DNA molecule contains a transmembrane-deleted gB fragment or derivative (e.g, gB_{680} or gBΔtm) and the pp65 antigens.

In the construction of the DNA molecules of the invention, one of skill in the art can readily select appropriate regulatory sequences, enhancers, suitable promoters, secretory signal sequences and the like. In the examples below, the plasmids have been provided with a tetracycline repressor from *E. coli*. However, if desired, the plasmid or other DNA molecule may be engineered to contain another regulatable promoter, which "turns on" expression upon administration of an appropriate agent (e.g., tetracycline), permitting regulation of *in vivo* expression of the HCMV gene product. Such agents are well known to those of skill in the art. The techniques employed to insert the HCMV gene into the DNA molecule and make other alterations, e.g., to insert linker sequences and the like, are known to one of skill in the art. See, e.g., Sambrook et al, "Molecular Cloning. A Laboratory Manual" (2d edition), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

In one embodiment, the DNA molecules of the invention are plasmids. One exemplary plasmid is pTet-gB. Construction of this plasmid is described in more detail below. Plasmid TetotTA-gB contains the gene from HCMV (the unique long (UL) 55) encoding the full-length gB subunit protein and a tetracycline regulatable HCMV-immediate early promoter which controls expression of gB. For convenience, the sequences of the HCMV gene fragment encoding the full-length gB protein which were used in the examples below are provided in Fig. 3A-3E [SEQ ID NO: 1 and 2]. As discussed herein, this invention is not limited to this strain of HCMV. pTet-gB has been found to be useful alone, and in conjunction with the other DNA

molecules of the invention, and particularly the pΔRC-pp65 plasmid described below. pTet-gB is also particularly useful for priming immune responses to subsequently administered HCMV immunogenic compositions and vaccines.

The pTetotTA-gB plasmid has been deposited pursuant to the Budapest Treaty, in the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, U.S.A. This deposit, designated ATCC 98029, was made on April 23, 1996 and is termed herein, pTet-gB.

Other plasmids provided herein, pΔRC-gB and pCBgB, also contain the HCMV gene encoding the gB protein. As demonstrated below, these DNA plasmids have been found to be highly potent immunogens for HCMV. See Examples 8 and 14.

Another plasmid of the invention, pΔRC-gB_{680} contains the portion of the HCMV gene encoding the N-terminal 680 amino acids of the gB protein and is capable of expressing this fragment *in vivo* or *in vitro*. This gB fragment is designated herein gB_{1-680}. As illustrated in Figure 3A-E [SEQ ID NO:2], the full-length gB subunit protein consists of 907 amino acids. This plasmid, which expresses a secreted form of gB, has been found to be a more potent immunogen than the plasmids expressing the full-length gB.

Also provided herein is plasmid pCDgBΔtm, which contains a deletion of the gB transmembrane region. This plasmid has been found to induce HCMV-specific neutralizing antibodies (see Example 14) and to be a more potent immunogen than the corresponding DNA plasmid encoding full-length gB.

Plasmid pΔRC-exon-4 plasmid contains the portion of the HCMV immediate-early (IE) gene encoding HCMV IE-exon-4 and is capable of expressing the gene product. The HCMV IE-exon-4 gene fragment has been described in

international patent application PCT/US94/02107, published August 18, 1994, which is incorporated by reference herein. The IE gene and the intron/exon junctions for Towne strain HCMV are provided in Stenberg et al, J. Virol., 49:190-199 (1984), and are available from GenBank under accession number K01484, M11828-30. The sequences of the IE-exon-4 gene fragment, Towne strain, are provided in Fig. 4A - B [SEQ ID NO: 3 and 4], for convenience. This invention is not limited to the use of the IE-exon-4 sequences from this viral strain.

Plasmid pΔRC-pp65 contains the HCMV gene encoding the HCMV phosphoprotein (pp) 65 tegument protein and is capable of expressing pp65 *in vivo* or *in vitro*. As described herein, immunization with pΔRC-pp65 induced a reduction of virus titers in the mouse lung after intranasal challenge with vaccinia recombinants carrying the pp65 gene, suggesting the protective function of cell-mediated immunity in lung after DNA immunization. Further, in contrast to a prior art pp65-containing plasmid construct which induced ELISA antibodies in only about 60% of inoculation mice, nearly 100% of mice inoculated with pΔRC-pp65 responded with pp65-specific ELISA antibodies. The sequences of the pp65 gene, Towne and AD169 strains, have been described in H. Pande et al, Virol., 181(1):220-228 (1991) and are provided in Fig. 5 [SEQ ID NO: 5 - 8] for convenience. pp65 sequences may be readily isolated using known techniques from other HCMV strains, or obtained from commercial sources. The strain from which the pp65 sequences are derived is not a limitation on the present invention.

Plasmid pΔRC-pp150 contains the portion of the HCMV gene encoding the HCMV pp150 tegument protein and is capable of expressing pp150 *in vivo* or *in vitro*. The sequences of the pp150 gene, Ad169 strain, have been described in G. Jahn et al, J. Virol., 61(5):1358-1367

(1987) and are provided in Fig. 6A - B for convenience [SEQ ID NO: 9 and 10]. pp150 sequences may be readily isolated using known techniques from another HCMV strain, or obtained from commercial sources. The strain from which the pp150 sequences are derived is not a limitation on the present invention.

The DNA molecules, and particularly the plasmids described herein, may be used for expression of the gB, gB_{1-680} fragment, pp65, pp150, or IE-exon-4 *in vitro*. The molecules are introduced by conventional means into the desired host cell [see, Sambrook et al, cited above]. Suitable host cells include, without limitation, bacterial cells, mammalian cells and cell lines, e.g., A549 (human lung carcinoma) or 293 (transformed human embryonic kidney) cells.

The host cell, once transfected with the recombinant plasmid (or other DNA molecule) of the present invention, is then cultured in a suitable medium, such as Minimal Essential Medium (MEM) for mammalian cells. The culture conditions are conventional for the host cell and allow the expressed HCMV protein, e.g., gB, to be produced either intracellularly, or secreted extracellularly into the medium. Conventional protein isolation techniques are employed to isolate the expressed subunit from the selected host cell or medium.

Alternatively, transfected host cells are themselves used as antigens, e.g., in *in vitro* immunological assays, such as enzyme-linked immunosorbent assays (ELISA). Such assay techniques are well known to those of skill in the art.

In yet another embodiment, one or more of the DNA molecules (e.g., plasmids) described herein may be used directly as immunogens in an immunogenic composition or directly for priming the immune response to a subsequently administered immunogenic or vaccine

composition. According to this embodiment of the invention, the DNA molecule (e.g., plasmid) containing the HCMV gene or gene fragment is introduced directly (i.e., as "naked DNA") into the animal by injection. The DNA molecule of the invention, when introduced into an animal, transfects the host's cells and produces the CMV protein in those cells. Methods of administering so-called 'naked DNA', are known to those of skill in the art. [See. e.g., J. Cohen, Science, 259:1691-1692 (March 19, 19930; E. Fynan et al, Proc. Natl. Acad. Sci., 90:11478-11482 (Dec. 1993); J. A. Wolff et al, Biotechniques, 11:474-485 (1991); International Patent Application PCT WO94/01139, which are incorporated by reference herein for purposes of described various 'naked DNA' delivery methods.]

The preparation of a pharmaceutically acceptable immunogenic composition, having appropriate pH, isotonicity, stability and other conventional characteristics is within the skill of the art. Currently, in a preferred embodiment, one or more of the recombinant plasmids (or other DNA molecules) of the invention is suspended in an acceptable carrier such as isotonic water, phosphate buffered saline, or the like. Optionally, although currently less preferred, such a composition may contain other components, such as adjuvants, e.g., aqueous suspensions magnesium hydroxides.

An effective amount of an immunogenic composition of the invention preferably contains between 10 μg and 10 mg, and preferably between about 80 μg and 150 μg of DNA of the invention per inoculation. Desirably, for each inoculation, the DNA of the invention is formulated in about 100 μl of a suitable carrier. In a particularly preferred embodiment, each patient is administered 100 μg DNA, which is administered three times at about 4 week

intervals. Alternatively, the dosage regimen involved in the method for immunizing with the recombinant DNA molecule (e.g., plasmid) of the present invention can be determined considering various clinical and environmental factors known to affect vaccine administration. For example, following a first administration of an immunogenic composition of the invention, boosters may be administered approximately 2- to 15-weeks later. These boosters may involve an administration of the same immunogenic composition as was first administered, or may involve administration of an effective amount of another immunogenic composition of the invention. Additional doses of the vaccines of this invention may also be administered where considered desirable by the physician.

In another aspect, the present invention provides a method of inducing HCMV-specific immune responses in an animal. The method involves administering to an animal an effective amount of an immunogenic composition containing one or more of the DNA molecules of the invention, as described above. The immunogenic composition is administered by any suitable route, including oral, nasal routes, subcutaneous and intraperitoneal. However, currently preferred are the intramuscular and intradermal routes of administration.

In a particularly preferred embodiment of this aspect, the method of inducing an HCMV-specific immune response of the invention involves the administration of one or more immunogenic compositions of the invention. These compositions may be formulated so as to contain a single DNA molecule of the invention, or may contain mixtures of the DNA molecules of the invention. In one desirable embodiment, the composition contains pΔRc-gB_{680} or pCBgBΔtm. In another desirable embodiment, the composition contains a plasmid containing pp65 according to the invention. As illustrated in the examples below,

administration of pΔRC-pp65 has been found to induce a potent HCMV-specific immune response. In another desirable embodiment of the invention, the combined administration of pTet-gB and pΔRC-pp65 invention (which may be formulated in a single composition, or preferably, administered separately) induces potent HCMV-specific ELISA and neutralizing antibodies to both proteins. In yet another desirable embodiment, the present invention provides a composition containing a chimeric plasmid which expresses pp65 and gB_{680} or gB. Yet another desired embodiment involves combined administration of pΔRC-gB_{680} and pΔRC-pp65.

In another aspect of this invention, a method of priming immune responses to a human cytomegalovirus immunogenic or vaccinal composition is provided. This method involves administering an immunogenic composition of the invention prior to administration of a second immunogenic or vaccinal composition. Desirably, an effective amount of an immunogenic composition of the invention, e.g., containing pTet-gB, is administered between about 4 and 15 weeks prior to administration of the immunogenic or vaccinal composition. The second immunogenic or vaccinal composition, for which the immune response is enhanced or primed by the method of the invention, may be an immunogenic composition of the invention or a conventional immunogenic or vaccine composition. For example, such a composition may contain one or more HCMV proteins (e.g., the isolated, purified gB protein described in the examples below), a whole virus (e.g., semipurified Towne strain HCMV virion), or recombinant HCMV viruses. Suitable recombinant viruses are well known to those of skill in the art and include, e.g., the Ad-gB virus [G. Marshall et al, (1990), cited above, and EP 389 286; the Ad-gB-IE-exon-4 virus [WO 94/17810]; the Ad-gB fragment viruses [WO 94/23744].

Other suitable HCMV vaccinal compositions are well known to those of skill in the art.

These examples illustrate the preferred methods for preparing and using the plasmids of the invention. These examples are illustrative only and do not limit the scope of the invention.

Example 1 - Construction of pTet-gB plasmid

The full-length HCMV-gB gene was obtained from the plasmid pAd-gB [Marshall et al., J. Infect. Dis., 162:1177-1181 (1990)] by XbaI-XbaI-digestion.

The full length HCMV-gB was inserted into the plasmid pUHD10-3 [Gossen and Bujard, Proc. Natl. Acad. Sci. USA, 12:5547-5551 (1992)]. This plasmid contains:

(a) a tetracycline regulatable promoter (HCMV minimal promoter, - 53 relative to the start site, with heptamerized tet-operon derived from the regulatory region of tet^R - gene of transposon -10);

(b) a multiple cloning site (including an XbaI site); and

(c) an SV40 polyadenylation signal downstream of the polycloning site.

After inserting the HCMV-gB (referred to as pTetO-gB), the plasmid was digested with Hind III followed by blunt-ending, then digested with PvuI and the fragment containing the tetracycline regulatable promoter-HCMV-gB-SV40 polyA signal sequences was isolated and inserted into the plasmid pUHD15-1 [Gossen and Bujard, cited above]. This latter plasmid (hereafter referred to as ptTA) contains the HCMV-IE promoter-enhancer which constitutively drives the tTAgene followed by the SV40 polyA signal. The tTA-gene codes for a fusion protein consisting of the tetracycline repressor from *E. coli* and the carboxy-terminal 130 amino acids of the herpes simplex virus protein 16 gene (HSV

VP-16). This fusion protein is a powerful transactivator of the tetracycline regulatable promoter of pTeto (which drives the HCMV-gB gene), because of the specific and high affinity attachment of the tetracycline repressor to the tetracycline operator sequences ensures the activation of transcription from the minimal HCMV promoter by the transactivator domain of HSV VP-16 gene (fused to the tetracycline repressor). The gene activation is specific for the pteto promoter. In the presence of low, non-toxic concentration of tetracycline (1 μg/ml or less), however, the transactivation is switched off, since tetracycline prevents the attachment of the tetracycline repressor to the teto sequences and no or very low gene expression is allowed (i.e., only the minimal HCMV promoter basal activity which is negligible in almost all cell types investigated so far).

To obtain the gB-expression plasmid regulatable by tetracycline, ptTA was cut just upstream of the HCMV-IE promoter/enhancer by *Xho*I, blunt-ended and cut with *Pvu*I. The large fragment containing the HCMV-IE promoter-enhancer-tTA fusion protein gene followed by the SV40 polyA signal and the *E. coli* sequences of the plasmid (i.e., the replication origin and the beta-lactamase genes) were isolated. This isolated fragment was ligated with the fragment of pUHD10-3 containing the gB gene by the competent blunt-end and PvuI ends, resulting in the plasmid pteto-gB-tTA. The resulting plasmid contains both the transactivator and the HCMV-gB gene. The structure of the plasmid is, in addition to the E.coli-part, tetracycline-regulatable promoter (7 teto + minimal HCMV promoter) followed by the HCMV-gB gene, followed by the SV40 polyA signal, followed by the HCMV-IE promoter-enhancer, followed by the tTA gene and ending with the SV40 polyA signal.

The tetracycline-controllable expression system has been found to work correctly *in vivo* in the mouse as well [J. Dhawan et al, Somatic Cell and Molecular Genetics, 21:233-240 (1995)]. The pTet-gB plasmid is suitable to control naked DNA immunization. It is possible to give tetracycline to mice in their drinking water in concentrations not toxic for the animals but reaching sufficient levels able to regulate expression in muscle tissues [J. Dhawan et al., Somatic Cell and Molecular Genetics, 21: 233-240 (1995)]. By tetracycline treatment of transfected cultures or inoculated mice the time of antigen exposure can be manipulated. The silent presence of the inoculated plasmid can be tested. Without tetracycline treatment, however, this plasmid simply serves as a plasmid DNA immunogen or vaccine.

Example 2 - Construction of further Plasmids

A. Construction of pRC-gB

pRC/CMV (Invitrogen Corporation) contains the HCMV-IE promoter. The full length gB gene (XbaI-XbaI fragment from pAd5-gB) was obtained using conventional techniques [SEQ ID NO:1] and inserted into pRC/CMV according to manufacturer's directions. The resulting plasmid is termed herein pRC-gB.

B. Construction of pΔRC-gB

pΔRC/CMV was derived from pRC/CMV plasmid by deleting the PvuII 1290 - PvuII 3557 fragment to obtain more unique restriction sites. The full gB [SEQ ID NO:1], derived from the plasmid pAd-gB [Marshall et al., J. Infect. Dis., 162:1177-1181 (1990)], was subcloned using conventional techniques, inserted into pUC-8 (commercially available), then obtained as a *Hind*III-*Bam*HI fragment and inserted into the *Hind*III-*Bam*HI digested pΔRC/CMV vector. The resulting plasmid is termed pΔRC-gB.

C. Construction of pΔRC-gB_{680}

pΔRC-gB_{680} expresses the N-terminal 680 amino acids of the gB protein [SEQ ID NO:2]. The plasmid was derived from pΔRC-gB, by deleting the C-terminal 227 amino acids of the gB by Xho-digestion, Klenow polymerase filling, removing the C-terminal portion of the gB gene, and religation of the 5400 bp fragment. The insert is approximately 2200bp.

Example 3 - Construction of pΔRC-pp65 and pΔRC-pp150

A. pΔRC-pp65

The plasmid pΔRC-pp65, which expresses the pp65 tegument protein of HCMV, was constructed as follows. H. Pande et al, Virology, 182(1):220-228 (1991), which provides the nucleotide sequences of the pp65 gene, is incorporated by reference herein [SEQ ID NO: 5 and 6].

The pp65 gene was isolated from the HCMV genome using conventional polymerase chain reaction techniques and inserted into a suitable expression plasmid. In this experiment, the 1696-bp pp65 gene was excised from the pUC-8-pp65 expression plasmid [Virogenetics] by *Nru*I - *Bam*HI digestion. The vector was blunt-ended with Klenow polymerase, digested with BamHI, and the pp65 gene inserted.

B. pΔRC-pp150

The plasmid, pΔRC-pp150, which expresses the pp150 tegument protein of HCMV, was constructed as follows. The pp150 gene was isolated from the HCMV genome using conventional polymerase chain reaction techniques and inserted into a suitable expression plasmid. One of skill in the art can readily isolate this gene from a desired HCMV strain making use of the published sequences in G. Jahn et al, J. Virol., 61(5):1358-1367 (1987) (which provides the nucleotide

sequences of the Ad169 HCMV pp150 gene and is incorporated by reference herein). See, also Fig. 6A-B herein [SEQ ID NO: 9 and 10].

In this experiment, the isolated HCMV-pp150 gene was inserted into the XbaI-restricted pΔRCd [Virogenetics]. The insert is approximately 3200 bp [SEQ ID NO: 10].

Example 4 - Construction of pΔRC-IE-Exon-4

The plasmid, pΔRC-IE-Exon-4, which expresses the HCMV-IE exon4 product [SEQ ID NO:4], was constructed as follow. The gene was obtained from pAd5-IE-Exon-4 [International Patent Application WO94/17810, published August 18, 1994 and Berencsi et al., Vaccine, 14:369-374 (1996)], by XbaI-digestion [SEQ ID NO:3]. The insert is 1230 bp.

Example 5 - Production of plasmid preparation stocks

E. coli DH5alfa competent cells (Gibco BRL, Gaithersburg, MD) were transformed with the constructed plasmids. Purified plasmid preparations were prepared on Plasmid Giga Kits (Qiagen Inc. Chatsworth, CA).

Example 6 - Expression of HCMV-proteins after transient transfection of 293 cells with the purified plasmid preparations

Transient transfections were performed by the purified plasmid preparations, 1.5 $\mu g/3x10^5$ cells, using lipofectamine (Gaithersburg, MD). Cells were tested for HCMV-protein expression 2 days after transfection by an immunofluorescence test as described in E. Gonczol et al, Science, 224:159-161 (1984). The antibodies used in this test include the monoclonal pp65-specific Ab [VIROSTAT, Portland, Maine, stock # 0831], monoclonal gB-specific Ab [Advanced Biotechnologies, Columbia, MD], and anti-pp150

monoclonal Ab [Virogenetics Corporation]. The IE-Exon-4-specific monoclonal Ab P63-27 was provided by W. Britt, University of Alabama at Birmingham.

The pTet-gB plasmid expresses the full-length HCMV-gB gene under the control of a tetracycline regulatable HCMV-IE promoter. The other plasmids express the inserted gene in transfected 293 cells under the control of the HCMV-IE promoter. Expression of gB, pp65 and pp150 was found to be strong using all plasmids.

After transfection with pTet-gB, 10-12% and <1% of cells expressed gB protein in the absence and presence, respectively, of 1μg tetracycline [Tetracycline hydrochloride, Sigma, St. Louis, MO]. Sixty to seventy percent and 40-50% of cells transfected with pΔRC-gB and $p\Delta gB_{680}$ plasmids, respectively, expressed gB. pp65 protein was expressed in 70-80% of cells transfected with pΔRC-pp65.

Example 7 - Immunization Procedures and Assay Methods

A. Immunization procedure

BALB/c or CBA mice were first pretreated i.m. with 100 μl of Bupivacaine HCl [0.25% Sensorcaine-MPF (ASTRA Pharmaceutical Products, Inc. Westborough, MA)]. In some experiments, identified below, no Bupivicaine pretreatment was used. One day later DNA was inoculated i.m. on the site of Bupivacaine infiltration. The dose for mice was 50-80 μg plasmid DNA/ inoculation. Booster inoculations were given i.m. 2x, without pretreatment with Bupivacaine. Mice immunized with pΔRC-gB plasmid were boosted 1 x. Mice were bled by retroorbital puncture at the indicated times.

B. ELISA

Semipurified HCMV virions and purified gB proteins may be prepared by immunoaffinity column chromatography as described in E. Gonczol et al, J.

Virol., 58:661-664 (1986). Alternatively, one of skill in the art can readily obtain suitable virions and gB proteins by alternative techniques.

Semipurified HCMV virions (Towne strain) or purified gB protein preparation were used as coating antigen for detection of gB-specific antibodies. OD values higher than mean OD values ± 2SD of preimmune sera were considered positive, or OD values >0.05, whichever was higher. Lysates of 293 cells transiently transfected with pARC-pp65 were used as coating antigen for detection of pp65-specific antibodies, lysates prepared from untransfected 293 cells served as control antigen. OD values obtained on control antigen-coated wells were subtracted from OD values obtained on pp65 antigen-coated wells and were considered positive if the resulting value was higher than 0.05.

C. Microneutralization assay

This assay was performed as described in E. Gonczol et al., J. Virol. Methods, 14:37-41 (1986). A neutralizing titer higher than 1:8 was considered positive.

D. Cytotoxic T lymphocyte assay

This assay was performed as described in K. Berencsi et al., J. Gen Virol., 74:2507-2512 (1993). Briefly, spleen cells of immunized mice were restimulated *in vitro* with VacWR-pp65-infected (m.o.i. = 0.2-0.5) autologous spleen cells (effector:stimulator ratio, 2.:1) for 5 days in 24-well plates. Cytolytic activity of nonadherent spleen cells was tested in a 4-h ^{51}Cr-release assay. Target cells (P815 MHC class I-matched, MC57 MHC class I-mismatched) were infected with VacWR-pp65 or VT-Vac WR (m.o.i. = 4-8). Percentage of specific ^{51}Cr-release was calculated as [(cpm experimental release -

cpm spontaneous release)/(cpm maximal release - cpm spontaneous release) x 100]. A pp65-specific cytotoxicity higher than 10% was considered positive.

Example 8 - Induction of HCMV-Specific Immune Responses by the Plasmid Constructs Expressing the gB Protein

BALB/c mice were inoculated 2 times at 0 and 5 weeks with 80 μg pΔRC-gB preparation. Serum samples at 5, 9 and 19 weeks after the first inoculation were tested for HCMV-specific ELISA antibodies and neutralizing antibodies (NA). The results are provided in Table 1 below, in which the ELISA antigen used was semipurified virions. The OD of responders is provided as the mean±SD at a serum dilution of 1:80. Mean ± 2SD of the 6 preimmunization sera at a dilution of 1:80 gave an OD value of 0.080. "GM" indicates the geometric mean.

Table 1

pΔRC-gB induces HCMV-specific ELISA and neutralizing antibodies (antigen: semipurified virion).

weeks after first inoculation	No. of ELISA responders/ total	OD of resp. dil 1:80	No. of NA resp. resp.	GM of NA
0	0/6	0.036±0.022	0/6	NA
5	5/6	0.314±0.188	2/2	19
9	6/6	1.387±0.810	6/6	34
19	ND	ND	4/4	22

These data demonstrate that all mice responded with both ELISA antibody and NA after the booster inoculation. The pΔRC-gB plasmid seems to be a highly potent immunizing construct.

Table 2

pTet-gB and pΔRC-pp65 induces insert-specific ELISA antibodies

Mice Immunized With:	Weeks after first Inoc.	# ELISA responders /total	OD* responders
pTet-gB	4	1/10	0.062
	8	9/10	0.277 ± 0.257
	13	7/7	0.530 ± 0.625
	21	6/6	0.503 ± 0.682
	31	5/6	0.451 ± 0.505
pΔRC-pp65	4	5/10	0.168 ± 0.070
	8	10/10	0.568 ± 0.387
	13	4/4	1.076 ± 0.216

* Mean OD ± SD of serum samples at dilution 1:40.

HCMV-specific ELISA antibodies were detected in 9 of 10 mice at 8 weeks after the first inoculation with pTet-gB (Table 2). HCMV neutralizing antibodies were detected in 4 of 10 mice, with titers between 1:16 and 1:48 (not shown). All mice immunized with the pΔRC-pp65 responded with pp65-specific ELISA antibodies. At 13 weeks (pp65- and gB-specific) and up to 31 weeks (gB-specific), OD values remained positive. In a separate experiment pp65-specific ELISA antibodies were also detected during the whole observation period (31 weeks) in 10 of the 10 immunized mice.

Example 9 - Induction of HCMV-Specific Immune Responses by the Plasmid Constructs Expressing pp65

To test whether the combination of the pTet-gB and pΔRC-pp65 results in reduced responses to the individual components, mice were immunized with both plasmids mixed together or inoculated separately. Groups of mice were inoculated with Bupivacaine (100 μl/mouse, 50 μl/leg),

and 2 days later, with either a mixture of both plasmids (80 μg of each DNA/mouse, 40 μg of each DNA/leg, 160 μg DNA/mouse) or each plasmid inoculated into two different legs (80 μg DNA of each plasmid/mouse, a total of 160 μg DNA/mouse inoculated in left and right legs). A similar booster was given 4 weeks later. The time course of both the gB- and pp65-specific ELISA antibody response was very similar in both groups, with nearly all mice developing antibodies by 8 or 13 weeks after the first inoculation (Table 3). In another experiment using the combination of the two plasmids, comparable OD values were observed up to 31 weeks after the first inoculation.

Table 3

pTet-gB and pΔRC-pp65 inoculated into the same animal induce gB and pp65-specific antibodies

Antigen, Inoculation	Weeks after 1st Inoc.	# gB-ELISA resp. /Total	OD* of responders	# pp65-ELISA resp. /Total	OD of Responders
pTet-gB+ pΔRC-pp65, mixed	4	4/10	0.087±0.024	5/10	0.078±0.033
	8	10/10	0.220±0.143	10/10	0.400±0.321
	13	10/10	0.392±0.152	9/10	0.303±0.224
pTet-gB+ pΔRC-pp65, separately	4	8/10	0.076±0.021	6/10	0.210±0.124
	8	9/10	0.202±0.268	8/10	0.452±0.333
	13	10/10	0.309±0.202	8/10	0.308±0.212

* The mean OD ± SD of serum samples at dilution 1:40.

Of six mice inoculated with pΔRC-pp65 alone at a single site, 3 mice responded with pp65-specific lysis of target cells (Fig. 2). In a second similar experiment, 3 of 9 mice immunized with pΔRC-pp65 alone showed strong

pp65-specific CTL responses. pp65-specific CTL were also detected in 4 of 5 tested mice inoculated with the mixture of pΔRC-pp65 and pTet-gB. When the pΔRC-pp65 and pTet-gB were inoculated separately into two different legs, 4 of 6 mice tested developed pp65-specific CTL response. These results establish that: 1) pp65-specific CTL responses are induced after DNA immunization; 2) there is no antigenic competition between the gB and pp65 proteins in the induction of antibody and CTL responses; and 3) gB protein expression in the cells at the inoculation site does not interfere with the presentation of pp65-specific T cell epitopes by MHC class I molecules to T cells.

Example 10 - Priming effect of pTet-gB

One inoculation of naked plasmid DNA in mice did not result significant antibody responses in a high percentage of mice. To find out whether the immune system of the nonresponder mice was specifically primed by the DNA inoculation, mice inoculated with pTet-gB were boosted 4 weeks later with either purified gB protein (5 μg gB/mouse in Alum s.c.) or with the Towne strain of HCMV (20 μg/mouse in Alum s.c.).

Table 4

Inoculation of mice with pTet-gB primes the immune system

Antigen	wks after priming	No. of NA responder/all	GM of NA/ responder
Teto-gB/*	4	0/10	5
Teto-gB	8	4/10	21
Teto-gB/*	4	0/10	4
gB+Alu	8	8/10	77
- / *	4	0/10	NA
gB+Alu	8	1/10	16
Teto-gB/**	12	1/5	16
Towne+Alu	14	5/5	97
- / **			
Towne+Alu	12	0/5	NA
	14	3/5	25

* second inoculations were given 4 weeks after the first inoculation
** Towne was given 12 weeks after the first inoculation

This data demonstrates that pTet-gB inoculation primes immune-responses. In other words, the combination of Teto-gB priming and gB+Alu or Towne+Alu booster gave higher number of responder mice and slightly higher NA titers than TetotTA-gB given 2 times.

Example 11 - DNA immunization decreases replication of the corresponding vaccinia recombinant in mice

Vaccinia virus recombinants expressing either HCMV-gB or pp65 were prepared using the methods described in WO 94/17810, published August 18, 1994. Briefly, the VacWR-gB and VacWR-pp65 recombinants were constructed as described [Gonczol et al, Vaccine, 9:631-637 (1991)], using the L variant of the neurovirulent WR strain of vaccinia virus as vector [Panicali et al, J. Virol.,

37(3):1000-1010 (1981)] and the gB or pp65 genes (HCMV Towne strain) as inserts cloned into the nonessential *Bam*HI site in the *Hind*III F region [Panicali and Paoletti, Proc. Natl. Acad. Sci., 79:4927-4931 (1982)] under the control of the vaccinia H6 early/late promoter. Vaccinia recombinant viruses and the parental wild-type WR strain were grown on Vero cells and purified as described [Gonczol et al, cited above].

After plasmid immunization, vaccinia virus recombinants expressing either HCMV-gB or pp65 were used for challenge in the model described in WO 94/23744, published October 27, 1994. Vaccinia virus WR strain replicates in mouse lung after intranasal inoculation and immune protection can be evaluated by virus titrations of the lung. Eight-week old female CBA and BALB/c mice were first pretreated with Bupivacaine, then 1 day later immunized either with pΔRC-gB or pΔRC-pp65 (80 μg/mouse). Mice were boosted 8 days later with DNA. Eight days after the second DNA dose mice were i.n. challenged either with $5x10^6$ pfu of Vaccinia WR-gB or Vaccinia WR-pp65. Lungs were taken at the time of virus challenge (day 0) and at days 1, 3, 4, 5, and 7 after challenge for virus titration. Lungs were homogenized, freeze-thaw 3 times and virus titer determined on Vero cells by plaque titration.

Table 5

Virus titers in the lungs of BALB/c mice immunized with pΔRC-gB or pΔRC-pp65 and challenged i.n. with Vac-gB

days after challenge	Vac-gB titer (log+SD) in lungs* pΔRC-gB-immunized	pΔRC-pp65-immunized	Diff. in titer (log)
0	3.29±2.83	3.29±2.83	0
1	2.24±2.9	2.76±2.51	-0.25
3	4.86±4.61	5.60±5.45	0.53
4	4.54±4.47	5.24±4.9	1.13
5	4.33±3.82	5.03±4.9	1.43
7	2.85±2.84	4.17±4.27	1.04

*Mean of titer (log) ± SD of 3 or 4 mice

Table 6

Virus titers in the lungs of BALB/c mice immunized with pΔRC-gB or pΔRC-pp65 and challenged i.n. with Vac-pp65

days after challenge	Vac - pp 65 titer (log±SD) in lungs* pΔRC-gB-immunized	pΔRC-pp65-immunized
0	5.52±4.83	5.52±4.83
1	4.31±4.3	4.56±3.5
3	7.68±6.75	7.15±7.11
4	7.7±7.66	6.57±6.56
5	7.45±6.79	6.02±6.14
7	7.17±6.17	6.23±6.08

*Mean of titer (log) ± SD of 3 or 4 mice

This data demonstrate that immunization with either plasmid reduced the titer of the corresponding challenge virus by 0.5-1.4 log on days 3, 4, 5 and 7 after the challenge.

Example 12 - Secreted form of gB is more potent immunogen than membrane-bound gB

To test whether gB bound to the membranes of gB-expressing cells or truncated form of gB lacking the transmembrane region of the molecule (it is secreted from the cell) induce stronger immune responses, mice were immunized with pΔRC-gB (expressing membrane-bound gB) or with $p\Delta RCgB_{680}$ (expressing the secreted form of gB) and ELISA and neutralizing antibody responses were evaluated as follows.

Plasmids pΔRC-gB (expressing the whole gB) and $\Delta RC\text{-}gB_{680}$ (expressing N-terminal 680 amino acids of the gB molecule and lacking the transmembrane region) were used in the following immunization protocol. Groups of 10 mice (BALB/c, female, 8 weeks old, purchased from HSD), were inoculated i.m. in the left leg with 50 μg plasmid DNA/mouse/inoculation. Mice were not inoculated with bupivacaine prior to DNA inoculation. Two months later a booster immunization was given (same dose, route).

Sera were tested in the gB-specific ELISA assay described above before the booster inoculation and 1 month after booster. The results are shown in Table 7, which shows the OD values of serum dilutions of 1:40 of individual mice. Preimmune serum samples of 40 mice were included. Cut off value: OD = 0.15.

Table 7

HCMV ELISA antibodies induced by plasmids expressing membrane-bound or secreted form of gB

OD of sera of mice immunized with					
pΔRC-gB			pΔRC-gB$_{680}$		
# of mouse	before booster	after booster	# of mouse	before booster	after booster
1	**0.31**	**0.55**	1	**0.83**	**>3.00**
2	0.09	0.10	2	**0.52**	**>3.00**
3	0.09	0.13	3	**1.65**	**>3.00**
4	0.06	0.08	4	0.06	0.09
5	0.07	0.07	5	**1.29**	**>3.00**
6	0.04	0.04	6	**1.92**	**>3.00**
7	0.08	**0.17**	7	**2.31**	**>3.00**
8	**0.51**	**1.88**	8	**1.22**	**>3.00**
9	0.07	0.07	9	**0.62**	**>3.00**
10	0.06	0.06	10	**1.50**	**>3.00**

The results in Table 7 show that ten mice immunized with the pΔRC-gB$_{680}$ were positive for stronger gB-specific antibody responses than mice immunized with pΔRC-gB.

Table 8 provides the results following the immunization protocol above, where the mice had been boosted after 2 months using the same protocol as described for the first immunization. Sera obtained 1 and 2 month after the booster were tested in a HCMV-microneutralization assay. Preimmune sera were included as negative controls, NA titers $\geq$ 12 are considered positive.

Table 8

pΔRC-gB_{680} expressing secreted form of gB induce stronger neutralizing antibody responses than pΔRC-gB expressing membrane-bound gB

NA titers of sera of mice 1 and 2 month after booster immunized with			
pΔRC-gB		pΔRC-gB680	
1M	2M	1M	2M
16	**24**	**128**	**64**
8	<8	**64**	**32**
4	<4	**256**	**192**
4	8	<4	**12**
8	4	**128**	**96**
4	4	**64**	**64**
8	**24**	**64**	**32**
48	**48**	**48**	**ND**
6	4	**96**	**96**
<6	4	**16**	**24**

As shown in Table 8, nine of the pΔRC-gB_{680}-immunized mice developed gB-specific antibodies, but only 3 of 10 responded in the pΔRC-gB-immunized group. HCMV-neutralizing antibody titers were also higher in the pΔRC-gB_{680}-immunized mice, 9 of 10 developed significant NA responses versus 3 of 10 in the pΔRC-gB-immunized group (Table 8).

These data show that the pΔRC-gB_{680} plasmid expressing the N-terminal 680 amino acids of gB (lacking the transmembrane region of the protein) given intramuscularly induces more potent antibody responses to gB than the pΔRC-gB plasmid expressing the full gB.

Example 13 - pΔRC-gB_{680} mixed with pΔRC-pp65 and given at one site or inoculated separately induce both gB- and pp65-specific antibodies

As shown above, pTet-gB and pΔRC-pp65 plasmids mixed and inoculated at one site induced immune responses to both gB and pp65 indicating that there is no antigenic competition between gB and pp65. In this experiment whether the pΔRC-gB_{680} (expressing the secreted form of gB) is suitable for immunization in a mixture with pΔRC-pp65 was tested.

Groups of 10 BALB/c mice (female, HSD, 9-10 weeks old) were inoculated either with a mixture of two plasmids containing 50 μg of each in 200 μl: 100 μl (50 μg) into the left leg, 100 μl (50 μg) into the right leg; or the two different plasmids were inoculated separately: one kind of DNA (100 μl/50 μg) into the left leg, the other kind of plasmid (100 μl/50 μg) into the right leg. A booster immunization was given 1 month later. The plasmids used in this study were pΔRC-pp65, pΔRC-gB, and pΔRC-gB_{680}. Table 9 shows results obtained with sera taken 8 days after booster. The ELISA antigen was purified gB. Cut off value: 0.081.

The results show that mice immunized with mixtures of pΔRC-gB and pΔRC-pp65 developed both gB and pp65 ELISA antibodies. Similar responses were observed in mice immunized with the two plasmids given at separate sites (Table 10 below). HCMV-gB-specific antibody responses in mice immunized with pΔRC-gB_{680} either given in mixture with pΔRC-pp65 or at separate sites were stronger than in mice immunized with the full-gB-expressing pΔRC-gB (these results confirm that the secreted form of gB is a stronger immunogen than the membrane-bound form).

Table 9

$p\Delta RC\text{-}gB_{680}$ mixed with $p\Delta RC$-pp65 and given at one site or inoculated separately induce gB-specific antibodies

gB-specific antibody (OD at serum dilutions of 1:40)							
mice inoculated with $p\Delta RC$-gB and $p\Delta RC$-pp65				mice inoculated with $p\Delta RC\text{-}gB_{680}$ and $p\Delta RC$-pp65			
mouse	at one site	mouse	at two sites	mouse	at one site	mouse	at two sites
#326	**0.085**	#356	**0.115**	#341	**1.280**	#336	**1.058**
#327	**0.193**	#357	**0.082**	#342	**1.070**	#337	**0.550**
#328	**0.121**	#358	**0.099**	#343	**1.385**	#338	**0.193**
#329	0.060	#359	**0.107**	#344	**1.190**	#339	**1.039**
#330	**0.115**	#360	**0.107**	#345	**2.588**	#340	**0.207**
#331	**0.093**	#361	NT	#351	**1.037**	#346	**0.288**
#332	0.061	#362	**0.092**	#352	**0.771**	#347	**0.220**
#333	**0.089**	#363	0.065	#353	**0.493**	#348	**0.513**
#334	0.078	#364	**0.152**	#354	**0.560**	#349	**0.223**
#335	**0.088**	#365	**0.082**	#355	**0.933**	#350	**0.719**
Mean OD:	**0.098**		**0.100**		**1.130**		**0.521**

Mice immunized as above with the mixture of $p\Delta RC\text{-}gB_{680}$ and $p\Delta RC$-pp65 showed gB-specific antibody responses similar to those observed in mice immunized with the two kinds of plasmids given at separate sites. Results of pp65-specific antibody responses showed that mice responded to the pp65 antigen regardless of immunization with a mixture or with plasmids given at separate sites (Table 10). Table 10 shows results obtained with sera taken 8 days after booster (cut off value: 0.050).

Table 10

$p\Delta RC\text{-}gB_{680}$ mixed with $p\Delta RC$-pp65 and given at one site or inoculated separately induce pp65-specific antibodies

	pp65-specific antibody (OD at serum dilutions of 1:40)							
	mice inoculated with $p\Delta RC\text{-}gB$ and $p\Delta RC$-pp65 pp65				mice inoculated with $p\Delta RC\text{-}gB_{680}$ and $p\Delta RC$-			
	mouse	at one site	mouse	at two sites	mouse	at one site	mouse	at two sites
	#326	0.037	#356	0.000	#341	**0.389**	#336	**0.276**
	#327	**0.149**	#357	0.000	#342	**0.238**	#337	**0.295**
	#328	0.002	#358	**0.508**	#343	**0.440**	#338	0.000
	#329	0.000	#359	0.008	#344	**0.077**	#339	0.009
	#330	0.009	#360	**0.176**	#345	0.008	#340	0.030
	#331	0.007	#361	dead	#351	**0.081**	#346	**0.051**
	#332	0.014	#362	0.009	#352	**0.077**	#347	**0.124**
	#333	0.000	#363	0.028	#353	0.049	#348	**0.281**
	#334	0.000	#364	**0.097**	#354	0.016	#349	**0.118**
	#335	0.008	#365	**0.201**	#355	**0.178**	#350	0.014
	Mean OD:	**0.014**		**0.109**		**0.154**		**0.111**

The data show that mice develop significant immune responses both to gB and pp65 after immunization with a mixture of $p\Delta RC\text{-}gB_{680}$ and $p\Delta RC$-pp65, indicating that these two HCMV antigens are able to induce parallel immune responses when introduced by expression plasmids to the immune system.

Example 14 - Immunization Studies in Mice Immunized with HCMV Plasmid Vectors Expressing Full-Length and Transmembrane-Deleted gB

As shown in the studies described above, full-length gB and transmembrane-deleted gB have been found to induce a strong and long-term antibody response when delivered by plasmid DNA. The following experiments provide further evidence of this effect.

A. pCBgB and pCB-gBΔtm

The gB open reading frame (ORF, nucleotides 1-2724) was obtained from the CMV Towne strain [SEQ ID NO: 1] using conventional techniques. The gBΔtm (transmembrane-deleted gB) was obtained from the wild type gene by deleting in frame the sequences coding for the hydrophobic transmembrane domain of the protein [nucleotides 2143 - 2316 were deleted from the gB ORF, SEQ ID NO:1]. These two coding sequences were introduced into the polylinker of the eukaryotic expression vector pCB11 corresponding to a commercially available pUC backbone with the HCMV IE1 promoter/enhancer sequences and the terminator sequences from the bovine growth hormone gene (Fig. 7A). The resulting plasmids, pCBgB and pCBgBΔtm expressing the full-length gB and its truncated version, respectively, are shown in Fig. 8. Protein expression from pCBgB and from pCBgBΔtm was confirmed by immunofluorescence and immunoprecipitation after transfection into cultured CHO-K1 cells. The immunoprecipitation experiment indicated that only pCBgBΔtm gave rise to a secreted form of gB which could be recovered from the cell culture medium.

B. Immunization

The study described below was performed with pCBgB and pCBgBΔtm in 6-8 week old female BALB/c mice. Anesthetized (xylazine + ketamine) mice (8 per group) received three administrations of 50 μg pCBgB or pCBgBΔtm

at three week intervals (days 0, 21 and 42) either intramuscularly (IM) or intradermally (ID). For IM administration, DNA in 50μl of saline was injected into the quadriceps with a Hamilton syringe equipped with a 20 gauge needle. For ID administration, DNA in a total volume of 100 μl of saline was injected into 5 sites of shaved dorsal skin with a pneumatic jet injector.

In each group, mice were labeled and bled on days 14 (following 1 injection), 35 (following 2 injections), 56 (following 3 injections), 116 and 202. The anti-urease IgG antibody response was followed by ELISA against recombinant gB produced in MRC5 cells infected with ALVAC-gB. The sera collected on days 116 and 202 were analyzed for hCMV neutralization in complement dependent microneutralization assay [Gonczol et al, cited above (1986)]. The data is provided in Table 11 and summarized in Fig. 9.

TABLE 11

INDIVIDUAL ELISA TITERS
IN MICE IMMUNIZED WITH HCMV GB PLASMID VECTORS

		Intramuscular		Intradermal		neg.
	#	pCBgB	pCBgBΔtm	pCBgB	pCBgBΔtm	serum
Day	Mouse	ELISA	ELISA	ELISA	ELISA	ELISA
14	1	50	50	<50	<50	<50
	2	<50	200	<50	<50	<50
	3	100	9600	100	<50	
	4	<50	300	<50	<50	
	5	100	100	<50	<50	
	6	<50	75	<50	50	
	7	100	75	<50	<50	
	8	50	<50	<50	<50	
35	1	100	100	75	50	<50
	2	150	900	150	600	<50
	3	200	12800	6400	2400	
	4	150	3200	1600	200	
	5	400	1200	100	1600	
	6	100	1200	1200	6400	
	7	150	300	75	100	
	8	150	100	200	150	

TABLE 11 (con't)

INDIVIDUAL ELISA TITERS
IN MICE IMMUNIZED WITH HCMV GB PLASMID VECTORS

		Intramuscular		Intradermal		neg.
Day	# Mouse	pCBgB ELISA	pCBgBΔtm ELISA	pCBgB ELISA	pCBgBΔtm ELISA	serum ELISA
56	1	150	1600	200	1200	<50
	2	200	2400	200	38400	<50
	3	200	38400	6400	12800	
	4	75	61200	6400	12800	
	5	400	2400	1200	4800	
	6	100	38400	3200	9600	
	7	200	19200	600	1600	
	8	600	4800	1200	4800	
116	1	<50	1200	75	600	<50
	2	1600	800	37.5	12800	<50
	3	400	9600	1200	640	
	4	<50	25600	2400	4800	
	5	25	1600	150	800	
	6	<50	25600	1600	4800	
	7	<50	6400	300	800	
	8	200	1200	200	800	
202	1	<50	1000	50	250	<50
	2	400	1000	25	8000	<50
	3	1600	8000	800	3000	
	4	<50	64000	1600	1500	
	5	25	1500	50	500	
	6	<50	24000	1200	3000	
	7	<50	4000	200	375	
	8		1000	150	375	

As illustrated in Table 11 above and in Fig. 9, pCBgB and pCBgBΔtm plasmids induced serum IgGs against recombinant gB protein after IM or ID administration in BALB/c mice [pCBgBΔtm/ID ≥ pCBgBΔtm/IM >> pCBgB/ID ≥ pCBgB/IM]. pCBgB and pCBgBΔtm plasmids induced detectable neutralizing antibodies to hCMV (*in vitro* assay) after IM or ID administration in BALB/c mice [pCBgBΔtm > pCBgB].

pCB-gB and pCB-gBΔtm have been observed to induce a strong and long-term antibody response. pCBgB and especially pCB-gBΔtm induce neutralizing antibodies.

The nature of the response (IgG_1/IgG_{2a}) differs between pCB-gB and pCB-gBΔtm. Particularly, pCB-gB has been observed to induce an IgG_1 (T_{H2}) response which is approximately equivalent to the IgG_{2a} (T_{H1}) response induced. In contrast, pCB-gBΔtm has been observed to induce an IgG_1 response that is significantly stronger that the IgG_{2a} response induced.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 8, lines 9-10 of the description

A. IDENTIFICATION OF DEPOSIT pTet-gB

Further deposits are identified on an additional sheet ☐

Name of depositary institution

American Type Culture Collection

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 USA

Date of deposit	Accession Number
April 23, 1996	98029

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

PAUL F. URRUTIA

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was

(Authorized Officer)

WHAT IS CLAIMED IS:

1. A DNA molecule which is non-replicating in mammals and comprises a sequence encoding a human cytomegalovirus antigen,

wherein the sequence is operably linked to regulatory sequences for expressing the antigen in mammals and wherein the antigen elicits an immune response in the mammal.

2. The DNA molecule according to claim 1 which is a plasmid.

3. The DNA molecule according to claim 1 or claim 2 wherein said antigen is selected from the group consisting of:

(a) gB;

(b) a gB derivative lacking at least the transmembrane domain;

(c) pp65;

(d) pp150;

(e) immediate-early exon-4; and

(f) combinations of (a) - (e).

4. The DNA molecule according to claim 3 which comprises a sequence encoding the gB and the pp65 antigens.

5. The DNA molecule according to claim 3 which comprises a sequence encoding the gB derivative and a sequence encoding the pp65 antigen.

6. A pTet-gB DNA plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gB gene and a tetracycline regulatable HCMV-immediate early promoter, said promoter controlling the expression of gB.

7. A pΔRC/CMV DNA plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gB gene and capable of expressing gB.

8. A pΔRC-gB_{680} plasmid, said plasmid comprising the portion of the human cytomegalovirus (HCMV) gene encoding the N-terminal 680 amino acids of the gB protein (gB_{1-680}) and capable of expressing gB_{1-680}.

9. A pΔRC-pp65 plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gene encoding the HCMV pp65 tegument protein and capable of expressing pp65.

10. A pΔRC-pp150 plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gene encoding the HCMV pp150 tegument protein and capable of expressing pp150.

11. A pΔRC-exon-4 plasmid, said plasmid comprising the portion of the human cytomegalovirus (HCMV) gene encoding HCMV immediate-early (IE)-exon-4 and capable of expressing IE-exon-4.

12. An immunogenic composition comprising a carrier and a DNA molecule according to any of claims 1-5.

13. The immunogenic composition according to claim 12 wherein the DNA molecule is selected from the group consisting of:

(a) pΔRC-gB;
(b) pTet-gB;
(c) pΔRC-pp65;
(d) pΔRC-gB_{680};
(e) pΔRC-pp150; and
(f) pΔRC-exon-4.

14. The immunogenic composition according to claim 12 or 13 comprising two or more DNA molecules.

15. The immunogenic composition according to claim 14 comprising a first DNA molecule which comprises a sequence encoding the gB antigen or a gB derivative, and a second DNA molecule which comprises a sequence encoding the pp65 antigen.

16. The immunogenic composition according to any of claims 12 to 15 wherein the carrier is selected from the group consisting of saline and isotonic water.

17. A method of inducing human cytomegalovirus-specific (HCMV) immune responses in an animal, comprising the step of administering to said animal an effective amount of a first immunogenic composition according to any of claims 12 to 16.

18. The method according to claim 17 wherein the composition comprises pTet-gB and pΔRC-pp65.

19. The method according to claim 16 further comprising the step of administering a second immunogenic composition to said animal, said second immunogenic composition comprising a plasmid selected from the group consisting of:

(a) pΔRC-gB;
(b) pTet-gB;
(c) pΔRC-pp65;
(d) pΔRC-gB_{680};
(e) pΔRC-pp150; and
(f) pΔRC-IE-Exon-4.

20. The method according to claim 17, wherein said second immunogenic composition is administered between about 2 to about 15 weeks following administration of said first immunogenic composition.

21. The use of a DNA molecule according to any of claims 1 to 5 or a plasmid according to any of claims 6 to 11 in the preparation of a medicament to treat a cytomegalovirus infection.

22. A method of priming immune responses to a selected human cytomegalovirus immunogenic composition, comprising the steps of:

administering a first immunogenic composition according to any of claims 12 to 16 and administering the selected human cytomegalovirus immunogenic composition.

23. The method according to claim 22 wherein the first immunogenic composition is administered between about 4 and 15 weeks prior to administration of the selected immunogenic composition.

24. The method according to claim 22 or claim 23 wherein the first immunogenic composition comprises pTet-gB.

25. The method according to claim 24, wherein pTet-gB is administered in an amount between about 50 μg to about 160 μg.

26. The method according to claim 22, wherein the selected immunogenic composition comprises an immunogen selected from the group consisting of a recombinant virus comprising an HCMV immunogen, an HCMV protein, and HCMV virions.

27. The method according to claim 26 wherein the HCMV protein is gB.

28. The method according to claim 26 wherein the recombinant virus is selected from the group consisting of Ad5.gb and Ad5-IE-exon-4.

AMENDED CLAIMS

[received by the International Bureau on 25 November 1997 (25.11.97);
original claims 13-28 replaced by new claims 13-32; remaining claims unchanged (4 pages)]

13. A pCBgBΔtm plasmid, said plasmid comprising the human cytomegalovirus (HCMV) gB gene containing a deletion in the transmembrane domain.

14. An immunogenic composition comprising a carrier and a DNA molecule according to any of claims 1-5.

15. The immunogenic composition according to claim 14 wherein the DNA molecule is selected from the group consisting of:

(a) pΔRC-gB;

(b) pTet-gB;

(c) pΔRC-pp65;

(d) pΔRC-gB_{680};

(e) pΔRC-pp150;

(f) pCBgB;

(g) pCBgBΔtm; and

(h) pΔRC-exon-4.

16. The immunogenic composition according to claim 14 or 15 comprising two or more DNA molecules.

17. The immunogenic composition according to claim 15 comprising a first DNA molecule which comprises a sequence encoding the gB antigen or a gB derivative, and a second DNA molecule which comprises a sequence encoding the pp65 antigen.

18. The immunogenic composition according to any of claims 13 to 17 wherein the carrier is selected from the group consisting of saline and isotonic water.

AMENDED SHEET (ARTICLE 19)

19. A method of inducing human cytomegalovirus-specific (HCMV) immune responses in an animal, comprising the step of administering to said animal an effective amount of a first immunogenic composition according to any of claims 14 to 18.

20. The method according to claim 19 wherein the composition comprises pTet-gB and pΔRC-pp65.

21. The method according to claim 19 further comprising the step of administering a second immunogenic composition to said animal, said second immunogenic composition comprising a plasmid selected from the group consisting of:

(a) pΔRC-gB;
(b) pTet-gB;
(c) pΔRC-pp65;
(d) pΔRC-gB_{680};
(e) pΔRC-pp150;
(f) pCBgB;
(g) pCBgBΔtm; and
(h) pΔRC-IE-Exon-4.

22. The method according to claim 19, wherein said second immunogenic composition is administered between about 2 to about 15 weeks following administration of said first immunogenic composition.

23. The use of a DNA molecule according to any of claims 1 to 5 or a plasmid according to any of claims 6 to 13 in the preparation of a medicament to treat a cytomegalovirus infection.

24. A method of priming immune responses to a selected human cytomegalovirus immunogenic composition, comprising the steps of:

administering a first immunogenic composition according to any of claims 14 to 18 and administering the selected human cytomegalovirus immunogenic composition.

25. The method according to claim 24 wherein the first immunogenic composition is administered between about 4 and 15 weeks prior to administration of the selected immunogenic composition.

26. The method according to claim 24 or claim 25 wherein the first immunogenic composition comprises pTet-gB.

27. The method according to claim 26, wherein pTet-gB is administered in an amount between about 50 μg to about 160 μg.

28. The method according to claim 24, wherein the selected immunogenic composition comprises an immunogen selected from the group consisting of a recombinant virus comprising an HCMV immunogen, an HCMV protein, and HCMV virions.

29. The method according to claim 28 wherein the HCMV protein is gB.

30. The method according to claim 28 wherein the recombinant virus is selected from the group consisting of Ad5.gb and Ad5-IE-exon-4.

31. A DNA molecule which is non-replicating in mammals and comprises a sequence encoding a human cytomegalovirus antigen selected from the group consisting of:

(a) pp65;

(b) pp150;

(c) immediate-early exon-4;

(d) gB and an antigen of (a) to (c);

(e) a gB derivative lacking at least the transmembrane domain and an antigen of (a) to (c); and

(f) a combination of antigens (a) to (c).

32. An immunogenic composition comprising a carrier and at least two DNA molecules, wherein said DNA molecules are selected from the group consisting of:

(a) gB;

(b) a gB derivative lacking at least the transmembrane domain;

(c) pp65;

(d) pp150; and

(e) immediate-early exon-4.

STATEMENT UNDER ARTICLE 19

Following entry of the attached replacement pages, claims 1 - 32 are pending.

The claims have been amended to recite two plasmid constructs disclosed throughout the application, but not previously claimed. See, new claims 12 and 13, amended claims 15 and 21. Particularly, pCBgB and pCBgBΔtm are supported in Example 14, page 34, line 1 through page 37, line 10 of the specification as filed. No new matter is added by this amendment. Inclusion of these two claims requires addition of two additional inventors, as requested in the attached letter.

In addition, two new independent claims have been added, which are supported by original claims 1 and 12, but are of narrower scope. These claims are believed to overcome the prior art cited in the International Search Report.

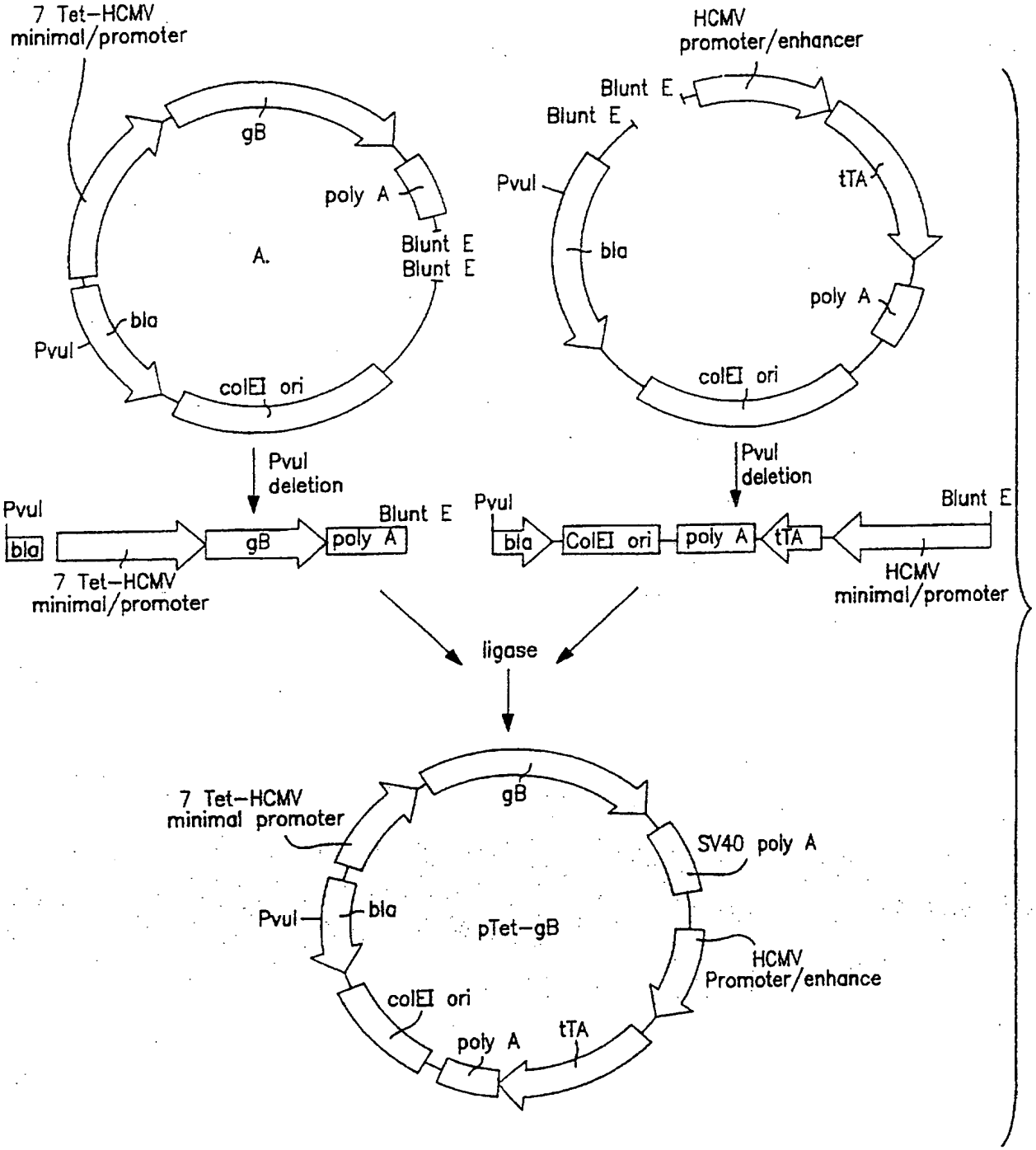

FIG. 1

SUBSTITUTE SHEET (RULE 26)

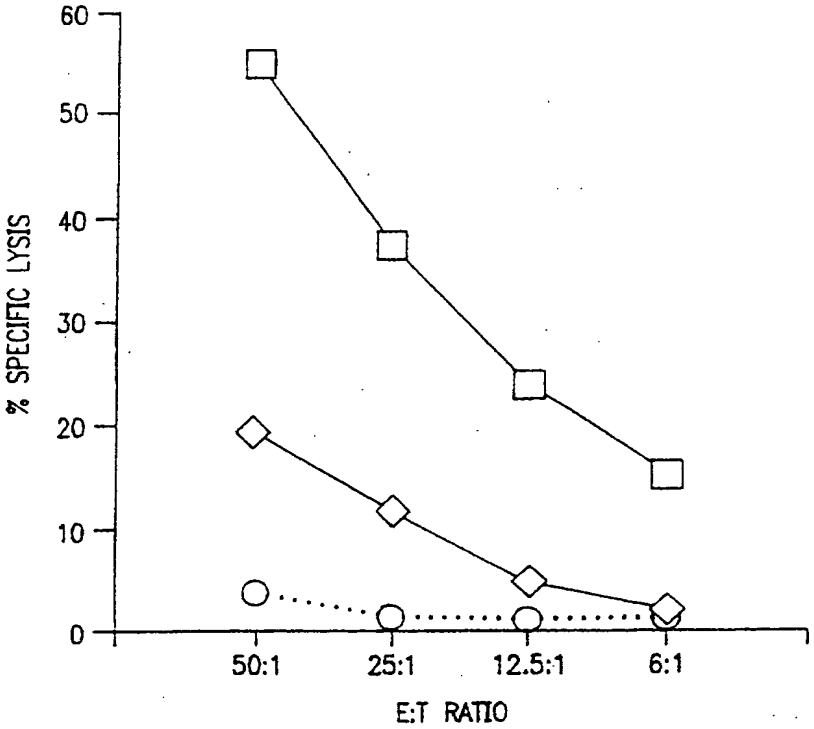

FIG. 2

FIGURE 3A

```
ATG GAA TCC AGG ATC TGG TGC CTG GTA GTC TGC GTT AAC TTG TGT          45
Met Glu Ser Arg Ile Trp Cys Leu Val Val Cys Val Asn Leu Cys
 1               5                  10                  15

ATC GTC TGT CTG GGT GCT GCG GTT TCC TCA TCT TCT ACT CGT GGA          90
Ile Val Cys Leu Gly Ala Ala Val Ser Ser Ser Ser Thr Arg Gly
            20                  25                  30

ACT TCT GCT ACT CAC AGT CAC CAT TCC TCT CAT ACG ACG TCT GCT         135
Thr Ser Ala Thr His Ser His His Ser Ser His Thr Thr Ser Ala
        35                  40                  45

GCT CAT TCT CGA TCC GGT TCA GTC TCT CAA CGC GTA ACT TCT TCC         180
Ala His Ser Arg Ser Gly Ser Val Ser Gln Arg Val Thr Ser Ser
    50                  55                  60

CAA ACG GTC AGC CAT GGT GTT AAC GAG ACC ATC TAC AAC ACT ACC         225
Gln Thr Val Ser His Gly Val Asn Glu Thr Ile Tyr Asn Thr Thr
65                  70                  75

CTC AAG TAC GGA GAT GTG GTG GGG GTC AAC ACC ACC AAG TAC CCC         270
Leu Lys Tyr Gly Asp Val Val Gly Val Asn Thr Thr Lys Tyr Pro
            80                  85                  90

TAT CGC GTG TGT TCT ATG GCA CAG GGT ACG GAT CTT ATT CGC TTT         315
Tyr Arg Val Cys Ser Met Ala Gln Gly Thr Asp Leu Ile Arg Phe
        95                  100                 105

GAA CGT AAT ATC GTC TGC ACC TCG ATG AAG CCC ATC AAT GAA GAC         360
Glu Arg Asn Ile Val Cys Thr Ser Met Lys Pro Ile Asn Glu Asp
    110                 115                 120

CTG GAC GAG GGC ATC ATG GTG GTC TAC AAA CGC AAC ATC GTC GCG         405
Leu Asp Glu Gly Ile Met Val Val Tyr Lys Arg Asn Ile Val Ala
125                 130                 135

CAC ACC TTT AAG GTA CGA GTC TAC CAG AAG GTT TTG ACG TTT CGT         450
His Thr Phe Lys Val Arg Val Tyr Gln Lys Val Leu Thr Phe Arg
            140                 145                 150

CGT AGC TAC GCT TAC ATC CAC ACC ACT TAT CTG CTG GGC AGC AAC         495
Arg Ser Tyr Ala Tyr Ile His Thr Thr Tyr Leu Leu Gly Ser Asn
        155                 160                 165

ACG GAA TAC GTG GCG CCT CCT ATG TGG GAG ATT CAT CAT ATC AAC         540
Thr Glu Tyr Val Ala Pro Pro Met Trp Glu Ile His His Ile Asn
    170                 175                 180

AGT CAC AGT CAG TGC TAC AGT TCC TAC AGC CGC GTT ATA GCA GGC         585
Ser His Ser Gln Cys Tyr Ser Ser Tyr Ser Arg Val Ile Ala Gly
185                 190                 195

ACG GTT TTC GTG GCT TAT CAT AGG GAC AGC TAT GAA AAC AAA ACC         630
Thr Val Phe Val Ala Tyr His Arg Asp Ser Tyr Glu Asn Lys Thr
            200                 205                 210
```

FIGURE 3B

```
ATG CAA TTA ATG CCC GAC GAT TAT TCC AAC ACC CAC AGT ACC CGT      675
Met Gln Leu Met Pro Asp Asp Tyr Ser Asn Thr His Ser Thr Arg
                215                 220                 225

TAC GTG ACG GTC AAG GAT CAA TGG CAC AGC CGC GGC AGC ACC TGG      720
Tyr Val Thr Val Lys Asp Gln Trp His Ser Arg Gly Ser Thr Trp
            230                 235                 240

CTC TAT CGT GAG ACC TGT AAT CTG AAT TGT ATG GTG ACC ATC ACT      765
Leu Tyr Arg Glu Thr Cys Asn Leu Asn Cys Met Val Thr Ile Thr
            245                 250                 255

ACT GCG CGC TCC AAG TAT CCC TAT CAT TTT TTC GCA ACT TCC ACG      810
Thr Ala Arg Ser Lys Tyr Pro Tyr His Phe Phe Ala Thr Ser Thr
            260                 265                 270

GGT GAT GTG GTT GAC ATT TCT CCT TTC TAC AAC GGA ACT AAT CGC      855
Gly Asp Val Val Asp Ile Ser Pro Phe Tyr Asn Gly Thr Asn Arg
            275                 280                 285

AAT GCC AGC TAT TTT GGA GAA AAC GCC GAC AAG TTT TTC ATT TTT      900
Asn Ala Ser Tyr Phe Gly Glu Asn Ala Asp Lys Phe Phe Ile Phe
            290                 295                 300

CCG AAC TAC ACT ATC GTC TCC GAC TTT GGA AGA CCG AAT TCT GCG      945
Pro Asn Tyr Thr Ile Val Ser Asp Phe Gly Arg Pro Asn Ser Ala
            305                 310                 315

TTA GAG ACC CAC AGG TTG GTG GCT TTT CTT GAA CGT GCG GAC TCA      990
Leu Glu Thr His Arg Leu Val Ala Phe Leu Glu Arg Ala Asp Ser
                320                 325                 330

GTG ATC TCC TGG GAT ATA CAG GAC GAG AAG AAT GTT ACT TGT CAA     1035
Val Ile Ser Trp Asp Ile Gln Asp Glu Lys Asn Val Thr Cys Gln
            335                 340                 345

CTC ACT TTC TGG GAA GCC TCG GAA CGC ACC ATT CGT TCC GAA GCC     1080
Leu Thr Phe Trp Glu Ala Ser Glu Arg Thr Ile Arg Ser Glu Ala
            350                 355                 360

GAG GAC TCG TAT CAC TTT TCT TCT GCC AAA ATG ACC GCC ACT TTC     1125
Glu Asp Ser Tyr His Phe Ser Ser Ala Lys Met Thr Ala Thr Phe
            365                 370                 375

TTA TCT AAG AAG CAA GAG GTG AAC ATG TCC GAC TCT GCG CTG GAC     1170
Leu Ser Lys Lys Gln Glu Val Asn Met Ser Asp Ser Ala Leu Asp
            380                 385                 390

TGT GTA CGT GAT GAG GCC ATA AAT AAG TTA CAG CAG ATT TTC AAT     1215
Cys Val Arg Asp Glu Ala Ile Asn Lys Leu Gln Gln Ile Phe Asn
            395                 400                 405

ACT TCA TAC AAT CAA ACA TAT GAA AAA TAT GGA AAC GTG TCC GTC     1260
Thr Ser Tyr Asn Gln Thr Tyr Glu Lys Tyr Gly Asn Val Ser Val
            410                 415                 420
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FIGURE 3C

```
TTT GAA ACC ACT GGT GGT TTG GTG GTG TTC TGG CAA GGT ATC AAG   1305
Phe Glu Thr Thr Gly Gly Leu Val Val Phe Trp Gln Gly Ile Lys
                425                 430                 435

CAA AAA TCT CTG GTG GAA CTC GAA CGT TTG GCC AAC CGC TCC AGT   1350
Gln Lys Ser Leu Val Glu Leu Glu Arg Leu Ala Asn Arg Ser Ser
            440                 445                 450

CTG AAT CTT ACT CAT AAT AGA ACC AAA AGA AGT ACA GAT GGC AAC   1395
Leu Asn Leu Thr His Asn Arg Thr Lys Arg Ser Thr Asp Gly Asn
        455                 460                 465

AAT GCA ACT CAT TTA TCC AAC ATG GAG TCG GTG CAC AAT CTG GTC   1440
Asn Ala Thr His Leu Ser Asn Met Glu Ser Val His Asn Leu Val
    470                 475                 480

TAC GCC CAG CTG CAG TTC ACC TAT GAC ACG TTG CGC GGT TAC ATC   1485
Tyr Ala Gln Leu Gln Phe Thr Tyr Asp Thr Leu Arg Gly Tyr Ile
485                 490                 495

AAC CGG GCG CTG GCG CAA ATC GCA GAA GCC TGG TGT GTG GAT CAA   1530
Asn Arg Ala Leu Ala Gln Ile Ala Glu Ala Trp Cys Val Asp Gln
            500                 505                 510

CGG CGC ACC CTA GAG GTC TTC AAG GAA CTT AGC AAG ATC AAC CCG   1575
Arg Arg Thr Leu Glu Val Phe Lys Glu Leu Ser Lys Ile Asn Pro
        515                 520                 525

TCA GCT ATT CTC TCG GCC ATC TAC AAC AAA CCG ATT GCC GCG CGT   1620
Ser Ala Ile Leu Ser Ala Ile Tyr Asn Lys Pro Ile Ala Ala Arg
    530                 535                 540

TTC ATG GGT GAT GTC CTG GGT CTG GCC AGC TGC GTG ACC ATT AAC   1665
Phe Met Gly Asp Val Leu Gly Leu Ala Ser Cys Val Thr Ile Asn
545                 550                 555

CAA ACC AGC GTC AAG GTG CTG CGT GAT ATG AAT GTG AAG GAA TCG   1710
Gln Thr Ser Val Lys Val Leu Arg Asp Met Asn Val Lys Glu Ser
            560                 565                 570

CCA GGA CGC TGC TAC TCA CGA CCA GTG GTC ATC TTT AAT TTC GCC   1755
Pro Gly Arg Cys Tyr Ser Arg Pro Val Val Ile Phe Asn Phe Ala
        575                 580                 585

AAC AGC TCG TAC GTG CAG TAC GGT CAA CTG GGC GAG GAT AAC GAA   1800
Asn Ser Ser Tyr Val Gln Tyr Gly Gln Leu Gly Glu Asp Asn Glu
    590                 595                 600

ATC CTG TTG GGC AAC CAC CGC ACT GAG GAA TGT CAG CTT CCC AGC   1845
Ile Leu Leu Gly Asn His Arg Thr Glu Glu Cys Gln Leu Pro Ser
605                 610                 615

CTC AAG ATC TTC ATC GCC GGC AAC TCG GCC TAC GAG TAC GTG GAC   1890
Leu Lys Ile Phe Ile Ala Gly Asn Ser Ala Tyr Glu Tyr Val Asp
            620                 625                 630
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FIGURE 3D

```
TAC CTC TTC AAA CGC ATG ATT GAC CTC AGC AGC ATC TCC ACC GTC   1935
Tyr Leu Phe Lys Arg Met Ile Asp Leu Ser Ser Ile Ser Thr Val
                635                 640                 645

GAC AGC ATG ATC GCC CTA GAC ATC GAC CCG CTG GAA AAC ACC GAC   1980
Asp Ser Met Ile Ala Leu Asp Ile Asp Pro Leu Glu Asn Thr Asp
                650                 655                 660

TTC AGG GTA CTG GAA CTT TAC TCG CAG AAA GAA TTG CGT TCC AGC   2025
Phe Arg Val Leu Glu Leu Tyr Ser Gln Lys Glu Leu Arg Ser Ser
                665                 670                 675

AAC GTT TTT GAT CTC GAG GAG ATC ATG CGC GAG TTC AAT TCG TAT   2070
Asn Val Phe Asp Leu Glu Glu Ile Met Arg Glu Phe Asn Ser Tyr
                680                 685                 690

AAG CAG CGG GTA AAG TAC GTG GAG GAC AAG GTA GTC GAC CCG CTG   2115
Lys Gln Arg Val Lys Tyr Val Glu Asp Lys Val Val Asp Pro Leu
                695                 700                 705

CCG CCC TAC CTC AAG GGT CTG GAC GAC CTC ATG AGC GGC CTG GGC   2160
Pro Pro Tyr Leu Lys Gly Leu Asp Asp Leu Met Ser Gly Leu Gly
                710                 715                 720

GCC GCG GGA AAG GCC GTT GGC GTA GCC ATT GGG GCC GTG GGT GGC   2205
Ala Ala Gly Lys Ala Val Gly Val Ala Ile Gly Ala Val Gly Gly
                725                 730                 735

GCG GTG GCC TCC GTG GTC GAA GGC GTT GCC ACC TTC CTC AAA AAC   2250
Ala Val Ala Ser Val Val Glu Gly Val Ala Thr Phe Leu Lys Asn
                740                 745                 750

CCC TTC GGA GCC TTC ACC ATC ATC CTC GTG GCC ATA GCC GTC GTC   2295
Pro Phe Gly Ala Phe Thr Ile Ile Leu Val Ala Ile Ala Val Val
                755                 760                 765

ATT ATC ATT TAT TTG ATC TAT ACT CGA CAG CGG CGT CTC TGC ATG   2340
Tyr Leu Ile Tyr Thr Arg Gln Arg Arg Leu Cys Met Gln Pro Leu
                770                 775                 780

CAG CCG CTG CAG AAC CTC TTT CCC TAT CTG GTG TCC GCC GAC GGG   2385
Ile Ile Ile Gln Asn Leu Phe Pro Tyr Leu Val Ser Ala Asp Gly
                785                 790                 795

ACC ACC GTG ACG TCG GGC AAC ACC AAA GAC ACG TCG TTA CAG GCT   2430
Thr Thr Val Thr Ser Gly Asn Thr Lys Asp Thr Ser Leu Gln Ala
                800                 805                 810

CCG CCT TCC TAC GAG GAA AGT GTT TAT AAT TCT GGT CGC AAA GGA   2475
Pro Pro Ser Tyr Glu Glu Ser Val Tyr Asn Ser Gly Arg Lys Gly
                815                 820                 825

CCG GGA CCA CCG TCG TCT GAT GCA TCC ACG GCG GCT CCG CCT TAC   2520
Pro Gly Pro Pro Ser Ser Asp Ala Ser Thr Ala Ala Pro Pro Tyr
                830                 835                 840
```

FIGURE 3E

```
ACC AAC GAG CAG GCT TAC CAG ATG CTT CTG GCC CTG GTC CGT CTG   2565
Thr Asn Glu Gln Ala Tyr Gln Met Leu Leu Ala Leu Val Arg Leu
                845                 850                 855

GAC GCA GAG CAG CGA GCG CAG CAG AAC GGT ACA GAT TCT TTG GAC   2610
Asp Ala Glu Gln Arg Ala Gln Gln Asn Gly Thr Asp Ser Leu Asp
                860                 865                 870

GGA CAG ACT GGC ACG CAG GAC AAG GGA CAG AAG CCC AAC CTG CTA   2655
Gly Gln Thr Gly Thr Gln Asp Lys Gly Gln Lys Pro Asn Leu Leu
                875                 880                 885

GAC CGA CTG CGA CAC CGC AAA AAC GGC TAC CGA CAC TTG AAA GAC   2700
Asp Arg Leu Arg His Arg Lys Asn Gly Tyr Arg His Leu Lys Asp
                890                 895                 900

TCC GAC GAA GAA GAG AAC GTC TGA                              2724
Ser Asp Glu Glu Glu Asn Val
                905
```

FIGURE 4A

```
ATG AAA CAG ATT AAG GTT CGA GTG GAC ATG CTG CGG CAT AGA ATC      45
Met Lys Gln Ile Lys Val Arg Val Asp Met Leu Arg His Arg Ile
 1               5                  10                  15

AAG GAG CAC ATG CTG AAA AAA TAT ACC CAG ACG GAA GAG AAA TTC      90
Lys Glu His Met Leu Lys Lys Tyr Thr Gln Thr Glu Glu Lys Phe
            20                  25                  30

ACT GGC GCC TTT AAT ATG ATG GGA GGA TGT TTG CAG AAT GCC TTA     135
Thr Gly Ala Phe Asn Met Met Gly Gly Cys Leu Gln Asn Ala Leu
        35                  40                  45

GAT ATC TTA GAT AAG GTT CAT GAG CCT TTC GAG GAG ATG AAG TGT     180
Asp Ile Leu Asp Lys Val His Glu Pro Phe Glu Glu Met Lys Cys
    50                  55                  60

ATT GGG CTA ACT ATG CAG AGC ATG TAT GAG AAC TAC ATT GTA CCT     225
Ile Gly Leu Thr Met Gln Ser Met Tyr Glu Asn Tyr Ile Val Pro
65                  70                  75

GAG GAT AAG CGG GAG ATG TGG ATG GCT TGT ATT AAG GAG CTG CAT     270
Glu Asp Lys Arg Glu Met Trp Met Ala Cys Ile Lys Glu Leu His
            80                  85                  90

GAT GTG AGC AAG GGC GCC GCT AAC AAG TTG GGG GGT GCA CTG CAG     315
Asp Val Ser Lys Gly Ala Ala Asn Lys Leu Gly Gly Ala Leu Gln
        95                  100                 105

GCT AAG GCC CGT GCT AAA AAG GAT GAA CTT AGG AGA AAG ATG ATG     360
Ala Lys Ala Arg Ala Lys Lys Asp Glu Leu Arg Arg Lys Met Met
    110                 115                 120

TAT ATG TGC TAC AGG AAT ATA GAG TTC TTT ACC AAG AAC TCA GCC     405
Tyr Met Cys Tyr Arg Asn Ile Glu Phe Phe Thr Lys Asn Ser Ala
125                 130                 135

TTC CCT AAG ACC ACC AAT GGC TGC AGT CAG GCC ATG GCG GCA TTG     450
Phe Pro Lys Thr Thr Asn Gly Cys Ser Gln Ala Met Ala Ala Leu
            140                 145                 150

CAG AAC TTG CCT CAG TGC TCC CCT GAT GAG ATT ATG GCT TAT GCC     495
Gln Asn Leu Pro Gln Cys Ser Pro Asp Glu Ile Met Ala Tyr Ala
        155                 160                 165

CAG AAA ATA TTT AAG ATT TTG GAT GAG GAG AGA GAC AAG GTG CTC     540
Gln Lys Ile Phe Lys Ile Leu Asp Glu Glu Arg Asp Lys Val Leu
    170                 175                 180

ACG CAC ATT GAT CAC ATA TTT ATG GAT ATC CTC ACT ACA TGT GTG     585
Thr His Ile Asp His Ile Phe Met Asp Ile Leu Thr Thr Cys Val
185                 190                 195

GAA ACA ATG TGT AAT GAG TAC AAG GTC ACT AGT GAC GCT TGT ATG     630
Glu Thr Met Cys Asn Glu Tyr Lys Val Thr Ser Asp Ala Cys Met
            200                 205                 210
```

FIGURE 4B

```
ATG ACC ATG TAC GGG GGC ATC TCT CTC TTA AGT GAG TTC TGT CGG      675
Met Thr Met Tyr Gly Gly Ile Ser Leu Leu Ser Glu Phe Cys Arg
                215                 220                 225

GTG CTG TCC TGC TAT GTC TTA GAG GAG ACT AGT GTG ATG CTG GCC      720
Val Leu Ser Cys Tyr Val Leu Glu Glu Thr Ser Val Met Leu Ala
                230                 235                 240

AAG CGG CCT CTG ATA ACC AAG CCT GAG GTT ATC AGT GTA ATG AAG      765
Lys Arg Pro Leu Ile Thr Lys Pro Glu Val Ile Ser Val Met Lys
                245                 250                 255

CGC CGC ATT GAG GAG ATC TGC ATG AAG GTC TTT GCC CAG TAC ATT      810
Arg Arg Ile Glu Glu Ile Cys Met Lys Val Phe Ala Gln Tyr Ile
                260                 265                 270

CTG GGG GCC GAT CCT CTG AGA GTC TGC TCT CCT AGT GTG GAT GAC      855
Leu Gly Ala Asp Pro Leu Arg Val Cys Ser Pro Ser Val Asp Asp
                275                 280                 285

CTA CGG GCC ATC GCC GAG GAG TCA GAT GAG GAA GAG GCT ATT GTA      900
Leu Arg Ala Ile Ala Glu Glu Ser Asp Glu Glu Glu Ala Ile Val
                290                 295                 300

GCC TAC ACT TTG GCC ACC CGT GGT GCC AGC TCC TCT GAT TCT CTG      945
Ala Tyr Thr Leu Ala Thr Arg Gly Ala Ser Ser Ser Asp Ser Leu
                305                 310                 315

GTG TCA CCC CCA GAG TCC CCT GTA CCC GCG ACT ATC CCT CTG TCC      990
Val Ser Pro Pro Glu Ser Pro Val Pro Ala Thr Ile Pro Leu Ser
                320                 325                 330

TCA GTA ATT GTG GCT GAG AAC AGT GAT CAG GAA GAA AGT GAG CAG     1035
Ser Val Ile Val Ala Glu Asn Ser Asp Gln Glu Glu Ser Glu Gln
                335                 340                 345

AGT GAT GAG GAA GAG GAG GAG GGT GCT CAG GAG GAG CGG GAG GAC     1080
Ser Asp Glu Glu Glu Glu Glu Gly Ala Gln Glu Glu Arg Glu Asp
                350                 355                 360

ACT GTG TCT GTC AAG TCT GAG CCA GTG TCT GAG ATA GAG GAA GTT     1125
Thr Val Ser Val Lys Ser Glu Pro Val Ser Glu Ile Glu Glu Val
                365                 370                 375

GCC CCA GAG GAA GAG GAG GAT GGT GCT GAG GAA CCC ACC GCC TCT     1170
Ala Pro Glu Glu Glu Glu Asp Gly Ala Glu Glu Pro Thr Ala Ser
                380                 385                 390

GGA GGC AAG AGC ACC CAC CCT ATG GTG ACT AGA AGC AAG GCT GAC     1215
Gly Gly Lys Ser Thr His Pro Met Val Thr Arg Ser Lys Ala Asp
                395                 400                 405

CAG TAA                                                        1221
Gln
```

FIGURE 5A

```
GCC ATG GCA TCC GTA CTG GGT CCC ATT TCG GGG CAC GTG CTG AAA      45
    Met Ala Ser Val Leu Gly Pro Ile Ser Gly His Val Leu Lys
      1               5                  10
        ATA
        Ile

GCC GTG TTT AGT CGC GGC GAC ACG CCG GTG CTG CCG CAC GAG ACG      90
Ala Val Phe Ser Arg Gly Asp Thr Pro Val Leu Pro His Glu Thr
 15                  20                  25
                        T

CGA CTC CTG CAG ACG GGT ATC CAC GTG CGC GTG AGC CAG CCC TCG     135
Arg Leu Leu Gln Thr Gly Ile His Val Arg Val Ser Gln Pro Ser
 30                  35                  40
                                A

CTG ATC CTG GTG TCG CAG TAC ACG CCC GAC TCG ACG CCA TGC CAC     180
Leu Ile Leu Val Ser Gln Tyr Thr Pro Asp Ser Thr Pro Cys His
 45                  50                  55
        T     A

CGC GGC GAC AAT CAG CTG CAG GTG CAG CAC ACG TAC TTT ACG GGC     225
Arg Gly Asp Asn Gln Leu Gln Val Gln His Thr Tyr Phe Thr Gly
 60                  65                  70

                                          5' splice-donor
AGC GAG GTG GAG AAC GTG TCG GTC AAC[GTG CAC AAC CCC ACG GGC     270
Ser Glu Val Glu Asn Val Ser Val Asn|Val His Asn Pro Thr Gly
 75                  80               [  85

       3' splice-acceptor
CGG AGC ATC TGC CCC AGC CAA]GAG CCC ATG TCG ATC TAT GTG TAC     315
Arg Ser Ile Cys Pro Ser Gln|Glu Pro Met Ser Ile Tyr Val Tyr
 90                  95     ]           100
  A                     G

GCG CTG CCG CTC AAG ATG CTG AAC ATC CCC AGC ATC AAC GTG CAC     360
Ala Leu Pro Leu Lys Met Leu Asn Ile Pro Ser Ile Asn Val His
105                 110                 115

CAC TAC CCG TCG GCG GCC GAG CGC AAA CAC CGA CAC CTG CCC GTA     405
His Tyr Pro Ser Ala Ala Glu Arg Lys His Arg His Leu Pro Val
120                 125                 130

GCT GAC GCT GTG ATT CAC GCG TCG GGC AAG CAG ATG TGG CAG GCG     450
Ala Asp Ala Val Ile His Ala Ser Gly Lys Gln Met Trp Gln Ala
135                 140                 145

CGT CTC ACG GTC TCG GGA CTG GCC TGG ACG CGT CAG CAG AAC CAG     495
Arg Leu Thr Val Ser Gly Leu Ala Trp Thr Arg Gln Gln Asn Gln
150                 155                 160
```

FIGURE 5B

```
TGG AAA GAG CCC GAC GTC TAC TAC ACG TCA GCG TTC GTG TTT CCC      540
Trp Lys Glu Pro Asp Val Tyr Tyr Thr Ser Ala Phe Val Phe Pro
165                 170                 175

ACC AAG GAC GTG GCA CTG CGG CAC GTG GTG TGC GCG CAC GAG CTG      585
Thr Lys Asp Val Ala Leu Arg His Val Val Cys Ala His Glu Leu
180                 185                 190

GTT TGC TCC ATG GAG AAC ACG CGC GCA ACC AAG ATG CAG GTG ATA      630
Val Cys Ser Met Glu Asn Thr Arg Ala Thr Lys Met Gln Val Ile
195                 200                 205

GGT GAC CAG TAC GTC AAG GTG TAC CTG GAG TCC TTC TGC GAG GAC      675
Gly Asp Gln Tyr Val Lys Val Tyr Leu Glu Ser Phe Cys Glu Asp
210                 215                 220

GTG CCC TCC GGC AAG CTC TTT ATG CAC GTC ACG CTG GGC TCT GAC      720
Val Pro Ser Gly Lys Leu Phe Met His Val Thr Leu Gly Ser Asp
225                 230                 235

GTG GAA GAG GAC CTG ACG ATG ACC CGC AAC CCG CAA CCC TTC ATG      765
Val Glu Glu Asp Leu Thr Met Thr Arg Asn Pro Gln Pro Phe Met
240                 245                 250

CGC CCC CAC GAG CGC AAC GGC TTT ACG GTG TTG TGT CCC AAA AAT      810
Arg Pro His Glu Arg Asn Gly Phe Thr Val Leu Cys Pro Lys Asn
255                 260                 265

ATG ATA ATC AAA CCG GGC AAG ATC TCG CAC ATC ATG CTG GAT GTG      855
Met Ile Ile Lys Pro Gly Lys Ile Ser His Ile Met Leu Asp Val
270                 275                 280

GCT TTT ACC TCA CAC GAG CAT TTT GGG CTG CTG TGT CCC AAG AGC      900
Ala Phe Thr Ser His Glu His Phe Gly Leu Leu Cys Pro Lys Ser
285                 290                 295

ATC CCG GGC CTG AGC ATC TCA GGT AAC CTA TTG ATG AAC GGG CAG      945
Ile Pro Gly Leu Ser Ile Ser Gly Asn Leu Leu Met Asn Gly Gln
300                 305                 310
                                        G

CAG ATC TTC CTG GAG GTG CAA GCG ATA CGC GAG ACC GTG GAA CTG      990
Gln Ile Phe Leu Glu Val Gln Ala Ile Arg Glu Thr Val Glu Leu
315                 320                 325
                      A       C

CGT CAG TAC GAT CCC GTG GCT GCG CTC TTC TTT TTC GAT ATC GAC     1035
Arg Gln Tyr Asp Pro Val Ala Ala Leu Phe Phe Phe Asp Ile Asp
330                 335                 340
```

FIGURE 5C

```
TTG CTG CTG CAG CGC GGG CCT CAG TAC AGC GAA CAC CCC ACC TTC   1080
Leu Leu Leu Gln Arg Gly Pro Gln Tyr Ser Glu His Pro Thr Phe
345                 350                 355
                                          G

ACC AGC CAG TAT CGC ATC CAG GGC AAG CTT GAG TAC CGA CAC ACC   1125
Thr Ser Gln Tyr Arg Ile Gln Gly Lys Leu Glu Tyr Arg His Thr
360                 365                 370

TGG GAC CGG CAC GAC GAG GGT GCC GCC CAG GGC GAC GAC GAC GTC   1170
Trp Asp Arg His Asp Glu Gly Ala Ala Gln Gly Asp Asp Asp Val
375                 380                 385

TGG ACC AGC GGA TCG GAC TCC GAC GAG GAA CTC GTA ACC ACC GAG   1215
Trp Thr Ser Gly Ser Asp Ser Asp Glu Glu Leu Val Thr Thr Glu
390                 395                 400
                                    A

CGC AAG ACG CCC CGC GTT ACC GGC GGC GGC GCC ATG GCG GGC GCC   1260
Arg Lys Thr Pro Arg Val Thr Gly Gly Gly Ala Met Ala Gly Ala
405                 410                 415
                      C

TCC ACT TCC GCG GGC CGC AAA CGC AAA TCA GCA TCC TCG GCG ACG   1305
Ser Thr Ser Ala Gly Arg Lys Arg Lys Ser Ala Ser Ser Ala Thr
420                 425                 430

GCG TGC ACG GCG GGC GTT ATG ACA CGC GGC CGC CTT AAG GCC GAG   1350
Ala Cys Thr Ala Gly Val Met Thr Arg Gly Arg Leu Lys Ala Glu
435                 440                 445
            T
            Ser

TCC ACC GTC GCG CCC GAA GAG GAC ACC GAC GAG GAT TCC GAC AAC   1395
Ser Thr Val Ala Pro Glu Glu Asp Thr Asp Glu Asp Ser Asp Asn
450                 455                 460

GAA ATC CAC AAT CCG GCC GTG TTC ACC TGG CCG CCC TGG CAG GCC   1440
Glu Ile His Asn Pro Ala Val Phe Thr Trp Pro Pro Trp Gln Ala
465                 470                 475

GGC ATC CTG GCC CGC AAC CTG GTG CCC ATG GTT GCT ACG GTT CAG   1485
Gly Ile Leu Ala Arg Asn Leu Val Pro Met Val Ala Thr Val Gln
480                 485                 490

GGT CAG AAT CTG AAG TAC CAG GAG TTC TTC TGG GAC GCC AAC GAC   1530
Gly Gln Asn Leu Lys Tyr Gln Glu Phe Phe Trp Asp Ala Asn Asp
495                 500                 505
                                    A
```

FIGURE 5D

```
ATC TAC CGC ATC TTC GCC GAA TTG GAA GGC GTA TGG CAG CCC GCT   1575
Ile Tyr Arg Ile Phe Ala Glu Leu Glu Gly Val Trp Gln Pro Ala
510                 515                 520

GCG CAA CCC AAA CGT CGC CGC CAC CGG CAA GAC GCC TTG CCC GGG   1620
Ala Gln Pro Lys Arg Arg Arg His Arg Gln Asp Ala Leu Pro Gly
525                 530                 535

CCA TGC ATC GCC TCG ACG CCC AAA AAG CAC CGA GGT TGAGCCACCC    1666
Pro Cys Ile Ala Ser Thr Pro Lys Lys His Arg Gly
540                 545                 550

GCCGCGCACG CTTAGGACGA CTCTATAAAA ACCCACGTCC ACTCAGACAC        1716
     A G

GCGACTTTTG GCCGCCACAC CTGTCGCCGC TGCTATATTT GCGACAGTTG        1766
  A                       A

CCGGAACCCT TCCCGACCTC CCACGAAGAC CCGTTCACCT TTGCGCATCC        1816

CCTGACCCCC CCCCTCATCC CGCCTTCGCG ATG TCT CAG GCA TCG TCC TCG  1867
                                 Met Ser Gln Ala Ser Ser Ser
                                                 555
        T       C                   A

CCC GGT GAG GGA CCC TCG TCG GAA GCG GCC GCG ATC AGC GAG GCC   1912
Pro Gly Glu Gly Pro Ser Ser Glu Ala Ala Ala Ile Ser Glu Ala
    560                 565                 570

                  Hind III
GAA GCC GCC AGC GGA AGC TT                                   1932
Glu Ala Ala Ser Gly Ser
    575
```

FIGURE 6A

```
TAGATCACCG ATAGAAATTT ACACGAGGCC ACGCCGGCCG GCAACAGCCA          50

CTGGTTGCTG AGTACGATAA AGGGTAGCAC AGTAAGCGTG AGAAAATTAG         100

TAGAGTAGAG GTTGGTCATG TAAATGGTGG GCGTCGAATA GCCAAGCACG         150

CGATTCGTGA GCAGCTGCGT GATCAACACT ATGGCGTTAA GTGGACCGCC         200

CACGAAGATG ATGAATGTGT TGAGTACGGC TTCGGTGGTT CGAATGGCGA         250

ATAGCGGCCC TGTCATGTTG CAAGTGTCAT TGATGTGCGG AGGAGTGTTG         300

TTGCGGGTCT GGGCGGAACA GCACACGGGG CGAAAAAACA GAAGAAACAA         350

                                                  >>>>>>>
GTCAGCGGCG CTTAAAAGAA AACCGCGTAT CCGCCTCCGC TATTAAACTA         400

                          ---------->
CCCCCCCTCC CTCTAGGTGG GGCGCTCACC GAGTTGTGGA TGATGGTGTC         450

                          Sac II
CATCGTGGGC GAATAGCAGA CCGCGGGCGC AGTCCGGGGC GACGACGCTT         500

CCGGGTTCTG GAGAAAAGCC AGC ATG AGT TTG CAG TTT ATC GGT CTA CAG  550
                         Met Ser Leu Gln Phe Ile Gly Leu Gln
                           1               5

CGG CGC GAT GTG GTA GCC CTG GTC AAC TTT CTG CGC CAT CTC ACG    595
Arg Arg Asp Val Val Ala Leu Val Asn Phe Leu Arg His Leu Thr
 10                  15                  20

CAA AAG CCC GAC GTG GAT CTC GAG GCA CAC CCC AAG ATC CTG AAA    640
Gln Lys Pro Asp Val Asp Leu Glu Ala His Pro Lys Ile Leu Lys
 25                  30                  35

AAA TGT GGC GAA AAA CGC CTG CAC CGG CGT ACG GTG CTG TTC AAC    685
Lys Cys Gly Glu Lys Arg Leu His Arg Arg Thr Val Leu Phe Asn
 40                  45                  50

GAG CTC ATG CTT TGG TTG GGA TAC TAC CGC GAG CTG CGT TTT CAC    730
Glu Leu Met Leu Trp Leu Gly Tyr Tyr Arg Glu Leu Arg Phe His
 55                  60                  65

AAC CCC GAC CTC TCC TCA GTG CTC GAG GAG TTC GAG GTG CGT TGC    775
Asn Pro Asp Leu Ser Ser Val Leu Glu Glu Phe Glu Val Arg Cys
 70                  75                  80

GTG GCC GTG GCG CGT CGC GGC TAC ACT TAC CCG TTC GGT GAT CGT    820
Val Ala Val Ala Arg Arg Gly Tyr Thr Tyr Pro Phe Gly Asp Arg
 85                  90                  95
```

FIGURE 6B

```
                                                                Eco RI
GGT AAG GCG CGT GAC CAC CTG GCT GTG CTA GAC CGT ACC GAA TTC      865
Gly Lys Ala Arg Asp His Leu Ala Val Leu Asp Arg Thr Glu Phe
100                 105                 110

GAT ACG GAC GTG CGC CAC GAT GCC GAG ATC GTG GAA CGC GCG CTC      910
Asp Thr Asp Val Arg His Asp Ala Glu Ile Val Glu Arg Ala Leu
115                 120                 125

GTA AGC GCG GTC ATT CTG GCC AAG ATG TCG GTG CGC GAG ACG CTG      955
Val Ser Ala Val Ile Leu Ala Lys Met Ser Val Arg Glu Thr Leu
130                 135                 140

GTC ACA GCC ATC GGC CAG ACG GAA CCC ATC GCC TTT GTG CAC CTC     1000
Val Thr Ala Ile Gly Gln Thr Glu Pro Ile Ala Phe Val His Leu
145                 150                 155

AAG GAT ACG GAG GTG CAG CGC ATT GAA GAA AAC CTG GAG GGT GTG     1045
Lys Asp Thr Glu Val Gln Arg Ile Glu Glu Asn Leu Glu Gly Val
160                 165                 170

CGC CGT AAC ATG TTC TGC GTG AAA CCG CTC GAC CTT AAC CTG GAC     1090
Arg Arg Asn Met Phe Cys Val Lys Pro Leu Asp Leu Asn Leu Asp
175                 180                 185

CGG CAC GCC AAC ACG GCG CTG GTC AAC GCC GTC AAC AAG CTC GTG     1135
Arg His Ala Asn Thr Ala Leu Val Asn Ala Val Asn Lys Leu Val
190                 195                 200

TAC ACG GGC CGT CTC ATC ATG AAC GTG CGC AGG TCT TGG GAG GAG     1180
Tyr Thr Gly Arg Leu Ile Met Asn Val Arg Arg Ser Trp Glu Glu
205                 210                 215

CTG GAG CGC AAA TGT CTG GCG CGC ATT CAG GAG CGC TGC AAG CTG     1225
Leu Glu Arg Lys Cys Leu Ala Arg Ile Gln Glu Arg Cys Lys Leu
220                 225                 230

CTG GTC AAG GAG CTG CGC ATG TGC CTT TCC TTT GAT TCC AAC TAC     1270
Leu Val Lys Glu Leu Arg Met Cys Leu Ser Phe Asp Ser Asn Tyr
235                 240                 245

TGT CGC AAT ATC CTC AAG CAC GCC GTG GAA AAC GGC GAC TCG GCC     1315
Cys Arg Asn Ile Leu Lys His Ala Val Glu Asn Gly Asp Ser Ala
250                 255                 260

GAC ACG CTG TTG GAG CTG CTC ATC GAG GAC TTT GAT ATC TAC GTG     1360
Asp Thr Leu Leu Glu Leu Leu Ile Glu Asp Phe Asp Ile Tyr Val
265                 270                 275
```

FIGURE 6C

```
GAC AGC TTC CCA CAG TCG GCG CAC ACG TTT TTG GGC GCG CGC TCG  1405
Asp Ser Phe Pro Gln Ser Ala His Thr Phe Leu Gly Ala Arg Ser
280                 285                 290

CCG TCG TTG GAG TTT GAC GAT GAC GCC AAT CTC CTC TCG CTC GGC  1450
Pro Ser Leu Glu Phe Asp Asp Asp Ala Asn Leu Leu Ser Leu Gly
295                 300                 305

GGC GGT TCG GCC TTC TCG TCG GTA CCC AAG AAA CAT GTC CCC ACG  1495
Gly Gly Ser Ala Phe Ser Ser Val Pro Lys Lys His Val Pro Thr
310                 315                 320

CAG CCG CTG GAC GGC TGG AGC TGG ATC GCC AGT CCC TGG AAG GGA  1540
Gln Pro Leu Asp Gly Trp Ser Trp Ile Ala Ser Pro Trp Lys Gly
325                 330                 335

CAC AAA CCG TTC CGC TTC GAG GCC CAT GGT TCT CTG GCA CCG GCC  1585
His Lys Pro Phe Arg Phe Glu Ala His Gly Ser Leu Ala Pro Ala
340                 345                 350

GCC GAA GCC CAC GCT GCC CGT TCG GCG GCC GTC GGC TAT TAC GAC  1630
Ala Glu Ala His Ala Ala Arg Ser Ala Ala Val Gly Tyr Tyr Asp
355                 360                 365

GAA GAG GAA AAG CGT CGC GAG CGG CAG AAA CGG GTG GAC GAC GAG  1675
Glu Glu Glu Lys Arg Arg Glu Arg Gln Lys Arg Val Asp Asp Glu
370                 375                 380

GTG GTG CAG CGT GAG AAA CAG CAG CTG AAG GCT TGG GAG GAG AGG  1720
Val Val Gln Arg Glu Lys Gln Gln Leu Lys Ala Trp Glu Glu Arg
385                 390                 395
          Pst I
CAG CAG AAC CTG CAG CAA CGT CAG CAG CAA CCA CCG CCC CCG GCA  1765
Gln Gln Asn Leu Gln Gln Arg Gln Gln Gln Pro Pro Pro Pro Ala
400                 405                 410

CGT AAA CCG AGC GCC TCC CGG AGG CTC TTT GGC TCC AGT GCC GAT  1810
Arg Lys Pro Ser Ala Ser Arg Arg Leu Phe Gly Ser Ser Ala Asp
415                 420                 425

GAG GAC GAC GAC GAT GAT GAT GAC GAG AAA AAC ATC TTT ACG CCC  1855
Glu Asp Asp Asp Asp Asp Asp Asp Glu Lys Asn Ile Phe Thr Pro
430                 435                 440

ATC AAG AAA CCG GGA ACT AGC GGC AAG GGC GCC GCT AGT GGT GGC  1900
Ile Lys Lys Pro Gly Thr Ser Gly Lys Gly Ala Ala Ser Gly Gly
445                 450                 455

GGT GTT TCC AGC ATT TTC AGC GGC CTG TTA TCC TCG GGC AGT CAG  1945
Gly Val Ser Ser Ile Phe Ser Gly Leu Leu Ser Ser Gly Ser Gln
460                 465                 470
```

FIGURE 6D

```
AAA CCG ACC AGC GGT CCC TTG AAC ATC CCG CAA CAA CAA CAG CGT  1990
Lys Pro Thr Ser Gly Pro Leu Asn Ile Pro Gln Gln Gln Gln Arg
475                 480                 485

CAC GCG GCT TTC AGT CTC GTC TCC CCG CAG GTG ACC AAG GCC AGC  2035
His Ala Ala Phe Ser Leu Val Ser Pro Gln Val Thr Lys Ala Ser
490                 495                 500

CCG GGA AGG GTC CGT CGG GAC AGC GCG TGG GAC GTG AGG CCG CTC  2080
Pro Gly Arg Val Arg Arg Asp Ser Ala Trp Asp Val Arg Pro Leu
505                 510                 515

ACG GAG ACC AGA GGG GAT CTT TTC TCG GGC GAC GAG GAT TCC GAC  2125
Thr Glu Thr Arg Gly Asp Leu Phe Ser Gly Asp Glu Asp Ser Asp
520                 525                 530

AGC TCG GAT GGC TAT CCC CCC AAC CGT CAA GAT CCG CGT TTC ACC  2170
Ser Ser Asp Gly Tyr Pro Pro Asn Arg Gln Asp Pro Arg Phe Thr
535                 540                 545

GAC ACG CTG GTG GAC ATC ACG GAT ACC GAG ACG AGC GCC AAA CCG  2215
Asp Thr Leu Val Asp Ile Thr Asp Thr Glu Thr Ser Ala Lys Pro
550                 555                 560

CCC GTC ACC ACC GCG TAC AAG TTC GAG CAA CCG ACG TTG ACG TTC  2260
Pro Val Thr Thr Ala Tyr Lys Phe Glu Gln Pro Thr Leu Thr Phe
565                 570                 575

GGC GCC GGA GTT AAC GTT CCT GCT GGC GCC GGC GCT GCC ATC CTC  2305
Gly Ala Gly Val Asn Val Pro Ala Gly Ala Gly Ala Ala Ile Leu
580                 585                 590

ACG CCG ACG CCT GTC AAT CCT TCC ACG GCC CCC GCT CCG GCC CCG  2350
Thr Pro Thr Pro Val Asn Pro Ser Thr Ala Pro Ala Pro Ala Pro
595                 600                 605

ACA CCT ACC TTC GCG GGT ACC CAA ACC CCG GTC AAC GGT AAC TCG  2395
Thr Pro Thr Phe Ala Gly Thr Gln Thr Pro Val Asn Gly Asn Ser
610                 615                 620

CCC TGG GCT CCG ACG GCG CCG TTG CCC GGG GAT ATG AAC CCC GCC  2440
Pro Trp Ala Pro Thr Ala Pro Leu Pro Gly Asp Met Asn Pro Ala
625                 630                 635

AAC TGG CCG CGC GAA CGC GCG TGG GCC CTC AAG AAT CCT CAC CTG  2485
Asn Trp Pro Arg Glu Arg Ala Trp Ala Leu Lys Asn Pro His Leu
640                 645                 650

GCT TAC AAT CCC TTC AGG ATG CCT ACG ACT TCC ACG GCT TCT CAA  2530
Ala Tyr Asn Pro Phe Arg Met Pro Thr Thr Ser Thr Ala Ser Gln
655                 660                 665
```

FIGURE 6E

```
AAC ACC GTG TCC ACC ACC CCT CGG AGG CCG TCG ACT CCA CGC GCC  2575
Asn Thr Val Ser Thr Thr Pro Arg Arg Pro Ser Thr Pro Arg Ala
670                 675                 680

GCG GTG ACA CAA ACA GCG TCT CGG GAC GCC GCT GAT GAG GTT TGG  2620
Ala Val Thr Gln Thr Ala Ser Arg Asp Ala Ala Asp Glu Val Trp
685                 690                 695

                      Pst I
GCT TTA AGG GAC CAA ACT GCA GAG TCA CCG GTC GAA GAC AGC GAG  2665
Ala Leu Arg Asp Gln Thr Ala Glu Ser Pro Val Glu Asp Ser Glu
700                 705                 710

GAG GAA GAC GAC GAC TCC TCG GAC ACC GGC TCC GTC GTC AGC CTG  2710
Glu Glu Asp Asp Asp Ser Ser Asp Thr Gly Ser Val Val Ser Leu
715                 720                 725

GGA CAC ACA ACA CCG TCG TCC GAT TAC AAC AAC GAC GTC ATT TCG  2755
Gly His Thr Thr Pro Ser Ser Asp Tyr Asn Asn Asp Val Ile Ser
730                 735                 740

CCT CCC AGT CAG ACG CCC GAG CAG TCG ACG CCG TCC AGA ATA CGT  2800
Pro Pro Ser Gln Thr Pro Glu Gln Ser Thr Pro Ser Arg Ile Arg
745                 750                 755

AAA GCT AAG TTA TCG TCT CCA ATG ACG ACG ACA TCC ACG AGC CAG  2845
Lys Ala Lys Leu Ser Ser Pro Met Thr Thr Thr Ser Thr Ser Gln
760                 765                 770

AAA CCG GTG CTG GGC AAG CGA GTC GCG ACG CCG CAC GCG TCC GCC  2890
Lys Pro Val Leu Gly Lys Arg Val Ala Thr Pro His Ala Ser Ala
775                 780                 785

CGA GCG CAG ACG GTG ACG TCG ACG CCG GTT CAG GGA AGG CTA GAG  2935
Arg Ala Gln Thr Val Thr Ser Thr Pro Val Gln Gly Arg Leu Glu
790                 795                 800

AAA CAG GTG TCG GGC ACG CCG TCG ACG GTA CCC GCC ACG CTG TTG  2980
Lys Gln Val Ser Gly Thr Pro Ser Thr Val Pro Ala Thr Leu Leu
805                 810                 815

CAA CCT CAA CCG GCT TCG TCT AAA ACG ACG TCA TCA AGG AAC GTG  3025
Gln Pro Gln Pro Ala Ser Ser Lys Thr Thr Ser Ser Arg Asn Val
820                 825                 830

ACT TCT GGC GCG GGA ACC TCT TCC GCT TCT TCG GCT CGA CAG CCG  3070
Thr Ser Gly Ala Gly Thr Ser Ser Ala Ser Ser Ala Arg Gln Pro
835                 840                 845
```

FIGURE 6F

```
TCA GCC TCG GCG TCC GTT TTG TCG CCC ACG GAG GAT GAT GTC GTG  3115
Ser Ala Ser Ala Ser Val Leu Ser Pro Thr Glu Asp Asp Val Val
850                 855                 860

TCC CCC GCC ACA TCG CCG CTG TCC ATG CTT TCG TCA GCC TCT CCG  3160
Ser Pro Ala Thr Ser Pro Leu Ser Met Leu Ser Ser Ala Ser Pro
865                 870                 875

TCC CCG GCC AAG AGT GCC CCC CCG TCT CCG GTG AAA GGC CGG GGC  3205
Ser Pro Ala Lys Ser Ala Pro Pro Ser Pro Val Lys Gly Arg Gly
880                 885                 890

AGC CGC GTC GGT GTT CCT TCC TTG AAA CCT ACT TTG GGC GGC AAG  3250
Ser Arg Val Gly Val Pro Ser Leu Lys Pro Thr Leu Gly Gly Lys
895                 900                 905

GCG GTG GTA GGT CGA CCG CCC TCG GTC CCC GTG AGC GGT AGC GCG  3295
Ala Val Val Gly Arg Pro Pro Ser Val Pro Val Ser Gly Ser Ala
910                 915                 920

CCG GGT CGC CTG TCC GGC AGC AGC CGG GCC GCC TCG ACC ACG CCG  3340
Pro Gly Arg Leu Ser Gly Ser Ser Arg Ala Ala Ser Thr Thr Pro
925                 930                 935

ACG TAT CCC GCG GTA ACC ACC GTT TAC CCA CCG TCG TCT ACG GCC  3385
Thr Tyr Pro Ala Val Thr Thr Val Tyr Pro Pro Ser Ser Thr Ala
940                 945                 950

AAA AGC AGC GTA TCG AAT GCG CCG CCT GTG GCC TCC CCC TCC ATC  3430
Lys Ser Ser Val Ser Asn Ala Pro Pro Val Ala Ser Pro Ser Ile
955                 960                 965

CTG AAA CCG GGG GCG AGC GCG GCT TTG CAA TCA CGC CGC TCG ACG  3475
Leu Lys Pro Gly Ala Ser Ala Ala Leu Gln Ser Arg Arg Ser Thr
970                 975                 980

GGG ACC GCC GCC GTA GGT TCC CCC GTC AAG AGC ACG ACG GGC ATG  3520
Gly Thr Ala Ala Val Gly Ser Pro Val Lys Ser Thr Thr Gly Met
985                 990                 995

AAA ACG GTG GCT TTC GAC CTA TCG TCG CCC CAG AAG AGC GGT ACG  3565
Lys Thr Val Ala Phe Asp Leu Ser Ser Pro Gln Lys Ser Gly Thr
1000                1005                1010

GGG CCG CAA CCG GGT TCT GCC GGC ATG GGG GGC GCC AAA ACG CCG  3610
Gly Pro Gln Pro Gly Ser Ala Gly Met Gly Gly Ala Lys Thr Pro
1015                1020                1025

TCG GAC GCC GTG CAG AAC ATC CTC CAA AAG ATC GAG AAG ATT AAG  3655
Ser Asp Ala Val Gln Asn Ile Leu Gln Lys Ile Glu Lys Ile Lys
1030                1035                1040
```

FIGURE 6G

AAC ACG GAG GAA TAGTTAAGAA ACACACACGC AGACGTACTT TTTAATGAAA 3707
Asn Thr Glu Glu
1045

CCATCGGATA GTGACGTGTC GGGAAAGGAG GACGGACGGA GGGTCAGGGA 3757

TGGGGAGACG TGAGAAAGTT GTCCGCGGGC AATTGCATGT CGCCCAGAAA 3807

GAACGTGGTT GTTCCGGCGG CGTGCATCTG CCGAAACACC GTGTGGTGGT 3857

TGTACGAGTA CACGTTACCG TCGCCCTCGG TAATTTGATA CAACGTGGCG 3907

ATGGGGGTGC CCTGCGGGAT CACGATGGAA CGCGTGCGCG TCCACAGCGT 3957

GACTTTGAGC GGCTCGCCGC CGCGCCACAC GCTGAGCCCC GTGTAAAAGG 4007

CGTCCTCGTG TGGCAAGTTG GCCACCAAGA AACACCGGTC TGTGATCTGC 4057

ACGTAGCGCA AGTCCAACTC CACCGTCTGC CGCGGTTGCA CCCCGAAGTG 4107

GATATCGTAA GGCGCGTGCA CCGTGAGCGA AAACACGTTG GGCTCATTGA 4157

GAAGCGGACA GTTGAGCGCG TCGCCGCTAA AAAAGAGTGA CGGGTTGCGG 4207

CTGAATCGCA GGTCGTACCC GCGCTGCGCG CTCGTCAGCA GGTAGAAGGA 4257

AAAAGCGCGC GGCATGTTGC GCGCCGTGAT CTTGTCCGAG ACGCGGTGAC 4307

AGAAGGAGGT GGCCACGGTG CCCAGCAGTT GGCGCTGTTC CGCGTCCACG 4357

Eco RI
CATAGTGAAT CCACGTTGAC GGTGAAAATG AGACCCATGA ATTCGTACTG 4407

CACGTTTTTG GACGCGATCC ACGCTTCGTC CTCGCCGGGT AGCGCTGCCT 4457

CGTCGTCGTC CATCGTGCCG CGGAACTGCG CGAGGTAGCG CGTAATTTTT 4507

TTGTGTCCGT ACGTGGTTAC GCGCTTACTG ATCCAGGTCA GATGGTCCAC 4557

GCGACATAGC AGCGTCGCGC CATGCCGCGT GACGCTGACC CGTCCAAAGG 4607

GCGCCGCCTC CTCCAACCCC GCAACGCCGC TCGGAGCACC GCCGCAGCCC 4657

GGCTTTCCCG GCGTCGTGAA AGGCACGGCG TAATGCGGGC AGGCGTGCGG 4707

CACGAAGGGC ACCATGACCA GTTGTGTGTG CAGAAAACCG ATCTGCACCG 4757

CCTGCGACTG CCGCATGGTT TCCTCGTCGT AAACCGCCAT GGACGAGCAG 4807

AGCCCGCCCT TGGTGATGAG CGGTTGCAGC ACCACGGAGC TCTCGCTGGT 4857

FIGURE 6H

GGAGCAGAGC AGAAAGAAGA GCTCGGCGTA CGCCGCCTTG GGCGTCACCA 4907

CGTTGGACCA GTCGTACTTG TAGCCGCAGC CCTGCGTGTT GTTGTAAATG 4957

Hind III

ACGGGAAACG AGAGAAAGAT GCAGCCCTGC ACGTACGAAG CTTTCTCCGT 5007

CACGTTCGAG GCCGTGTTGT ACTGCTCGGT GATGGACACC AAGTACGACT 5057

CGTAGGCCGT CAGGTGCGAG GCCGAACGGT GAATCTTGGC GTGGCGCACG 5107

CAGCGACCGT AGTTGTCGCG GTCCGCGTCG CGTAGCGCTT CGATCCACGA 5157

GGTCACCACG TCCTGCGCCG GCAGACGATA GTCCTGCTCG GGGTCCATGT 5207

GGCGGCACAG CCGCAGGCGC TCTGCCAGTT GGCGAGGGAT ACCGTCGTGC 5257

GACCTTTTGA CCGCGGTGGT GCCTGTCGTC CTCGTCTCCC CTCCTTCGTT 5307

Bam HI

CTCCCTGTTT TCTCTTCTCT CATTCCCGGT CTCCGGATCC GCAGCCGCTA 5357

CCTCTTGCTC CGCGGTTTTC TCGCCCACCT CGCTCGTCGC TGTCGCCGCC 5407

ACCGCAGCGG CGGCGACGGA CGGCGGCGGT AACAACAGCT CCGTGAAGCT 5457

GACGAGCGGC AGCGGCGACG ACGGTGGCGG CGACGACACG GCGACGGTCA 5507

ACAGGGTCAC AAGCGTGGGT TTGTCCCCCA TAATCTGGTC GCCGCCACCG 5557

CCGTCGTTGC CGGTCCCCGT TTCCTCCGGC GTCGCGGTTT CCGCCGTCTC 5607

CGGATGAGCG GCCGCGGCGC GGGCTCGGCG TCCCGCCGTC CGAGACGGTG 5657

TATATAAACC GCGTCGGCCT CGCCGGCCCG AGCGCGCCGG GGAGAAGAAC 5707

CTCTTCCCGG GCCCCGCGTT CAAGACGGCG TGCCGTGACG CTCGATGGGT 5757

CCGCTTCATC AGACTGCGTA CGCTTTGGAG CGTCAGACCC AGGGCGCATG 5807

TAGCCGACTT GGAGGACTTT GCCGCCTTTT ATCGCACCCT CTCGGACAGT 5857

GAGCAGCAGG AGTTCGAGCA AGAAGCCGAA CTCGCCTCCC GCTCACAACG 5907

CGTGCAACAC CTGCGCGAGG CCCGGCGCCA GCTCAAGATG GACCTGATGT 5957

GTCACGGCGG TTGAAAACGC GCATGATCTC GCGAAGCCAT CTACGCGCCT 6007

GTCAGGGCGA TGACGACATC AGCGATGACG GCTCCTGATA CGCGCCGGCA 6057

FIGURE 6I

```
 Pst I
GCTGCAGCAC GTGGAGACGC TGCGTCGGTT TCTGCGCGGC GACAGCTGCT        6107

TTGTGCACGA TCTCCCGGGC ATGATGGACT ATCACGACGG GCTCTCGCGC        6157

CGTCAACAGC GTGCCTTTTG CCGCGCGAGT CGCGTGTTGA CGGACCCGGA        6207

                                ** ****                --
GCCCATCCAG AGCGAAGCGG AGGGGGAGAA TAAACAGTTT ACGGAGCACA        6257

------J                         ******                 ---
CACACAAAGT AGTCTCGTTT TTTATTAAAA GTGTCTTTGT ATTTCCCTAT        6307

---J
CTTGTGTTGC CCAACTGCTG TCAGGTCTCC GTAGATCGCT CCCGGGTGCC CGA    6360
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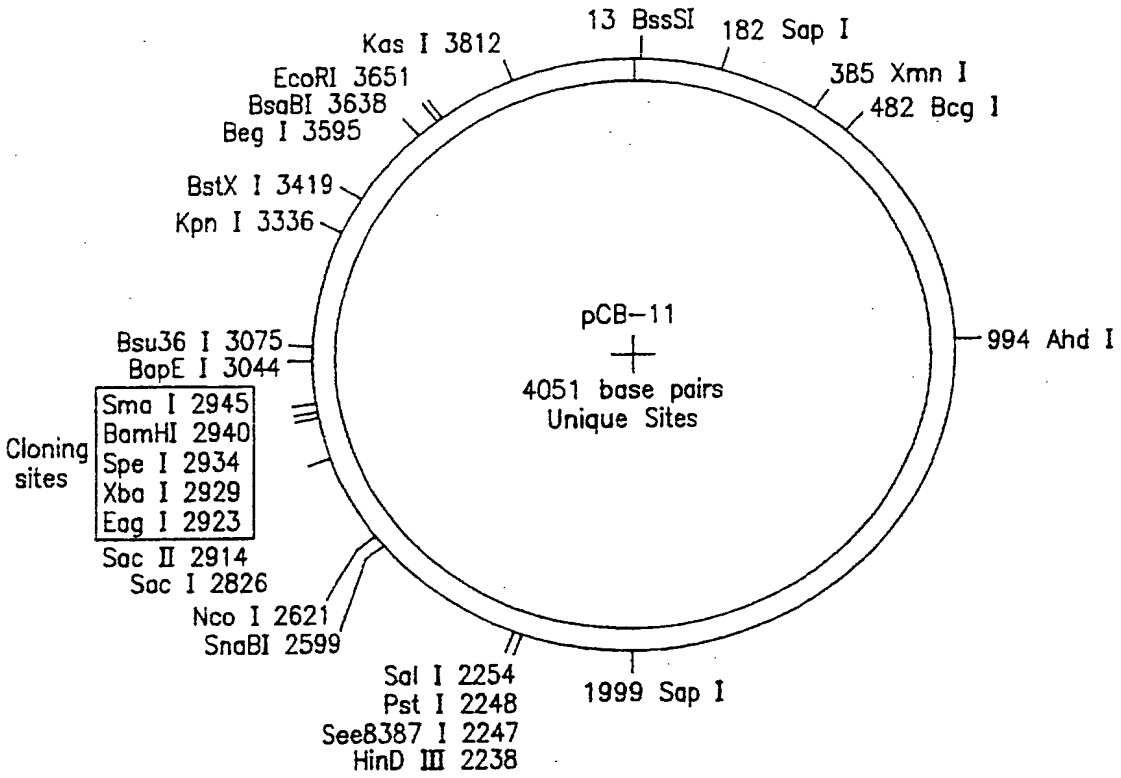

2255–2920: hCMV IE1 enhancer/promoter
2923–2951: Multiple cloning sites
2952–3650: BGH terminator
3651–4051 and 1–2254: pUC19

FIG. 7A

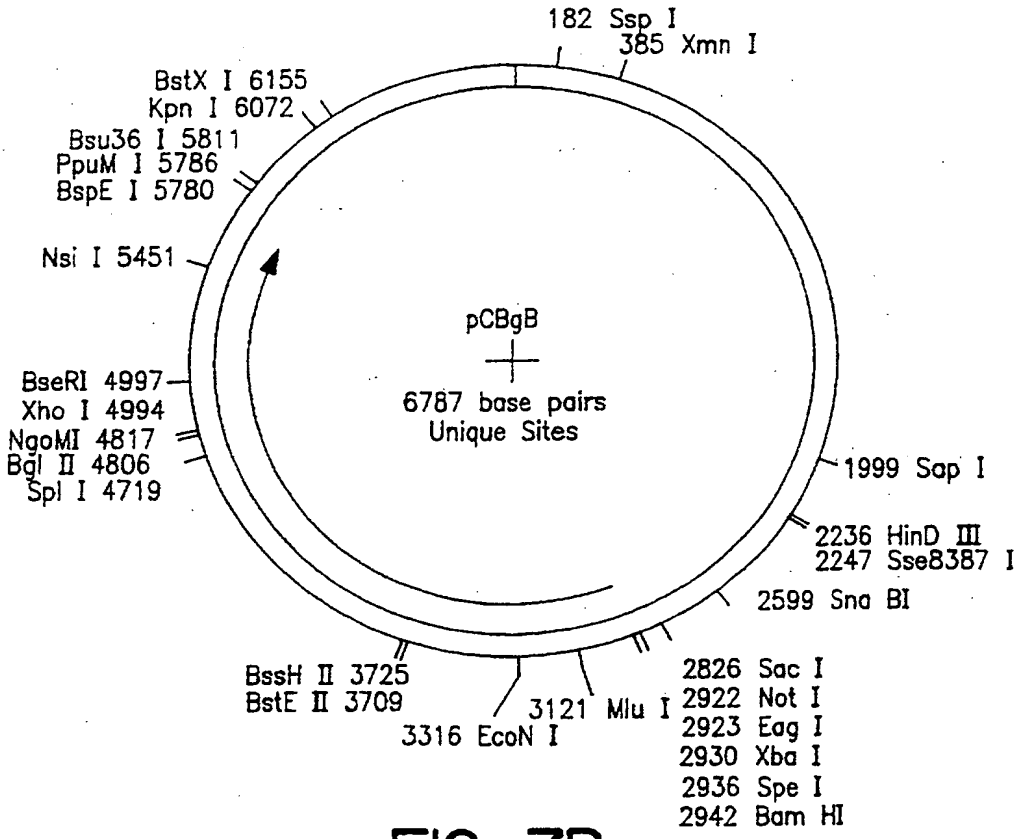

FIG. 7B

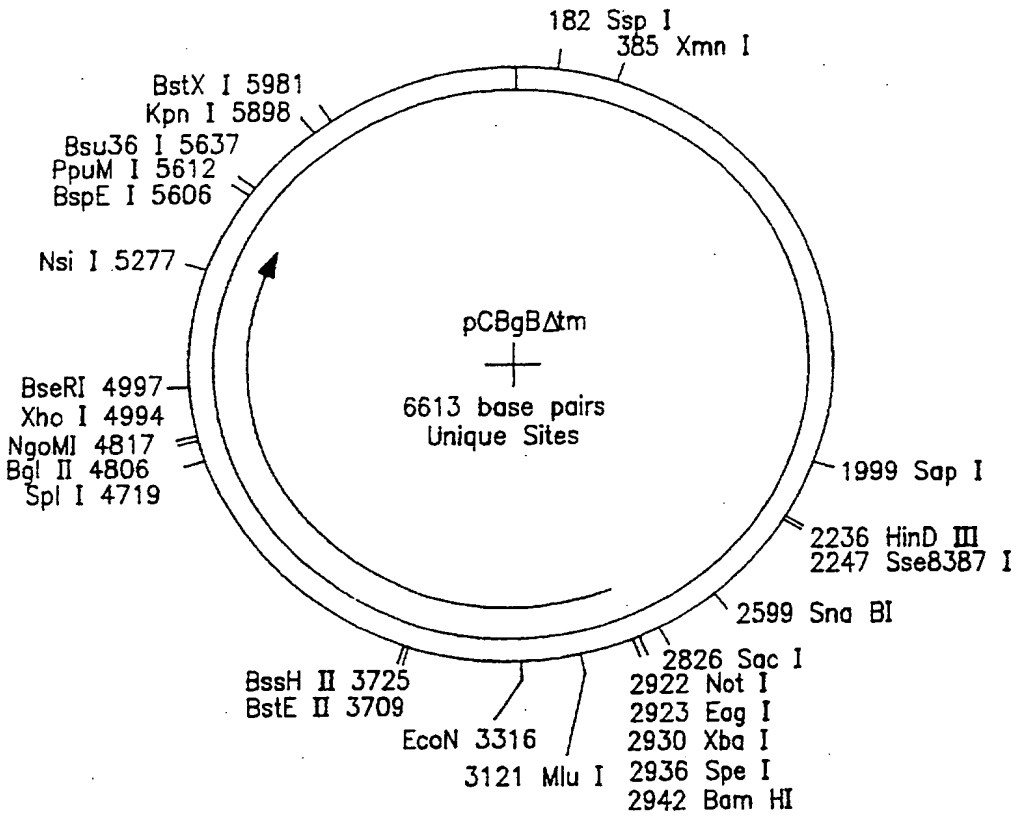

FIG. 7C

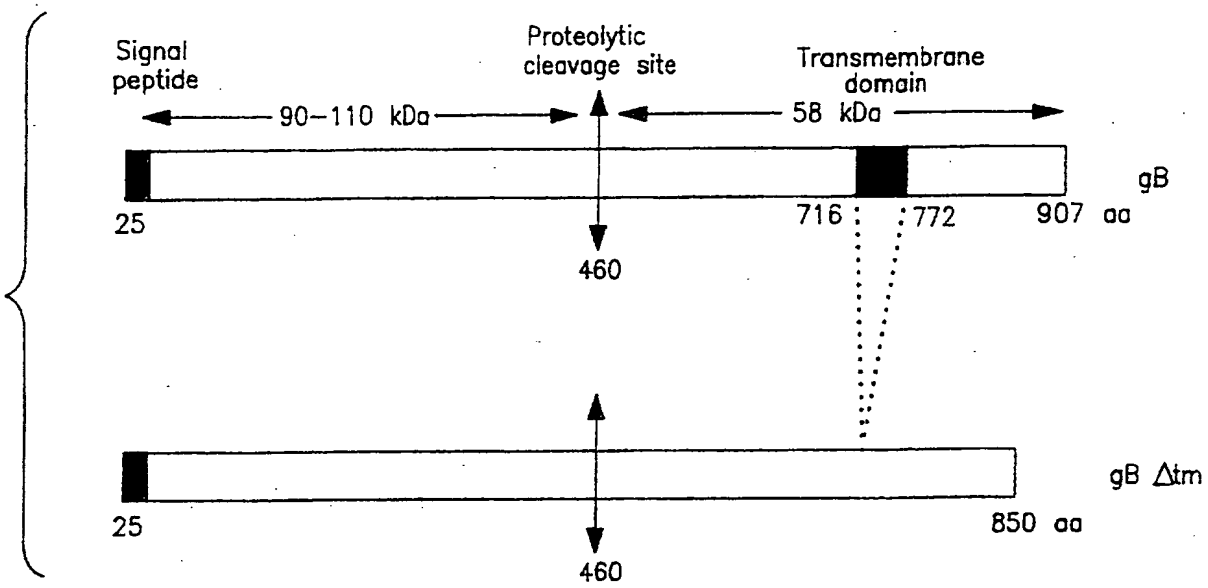

FIG. 8

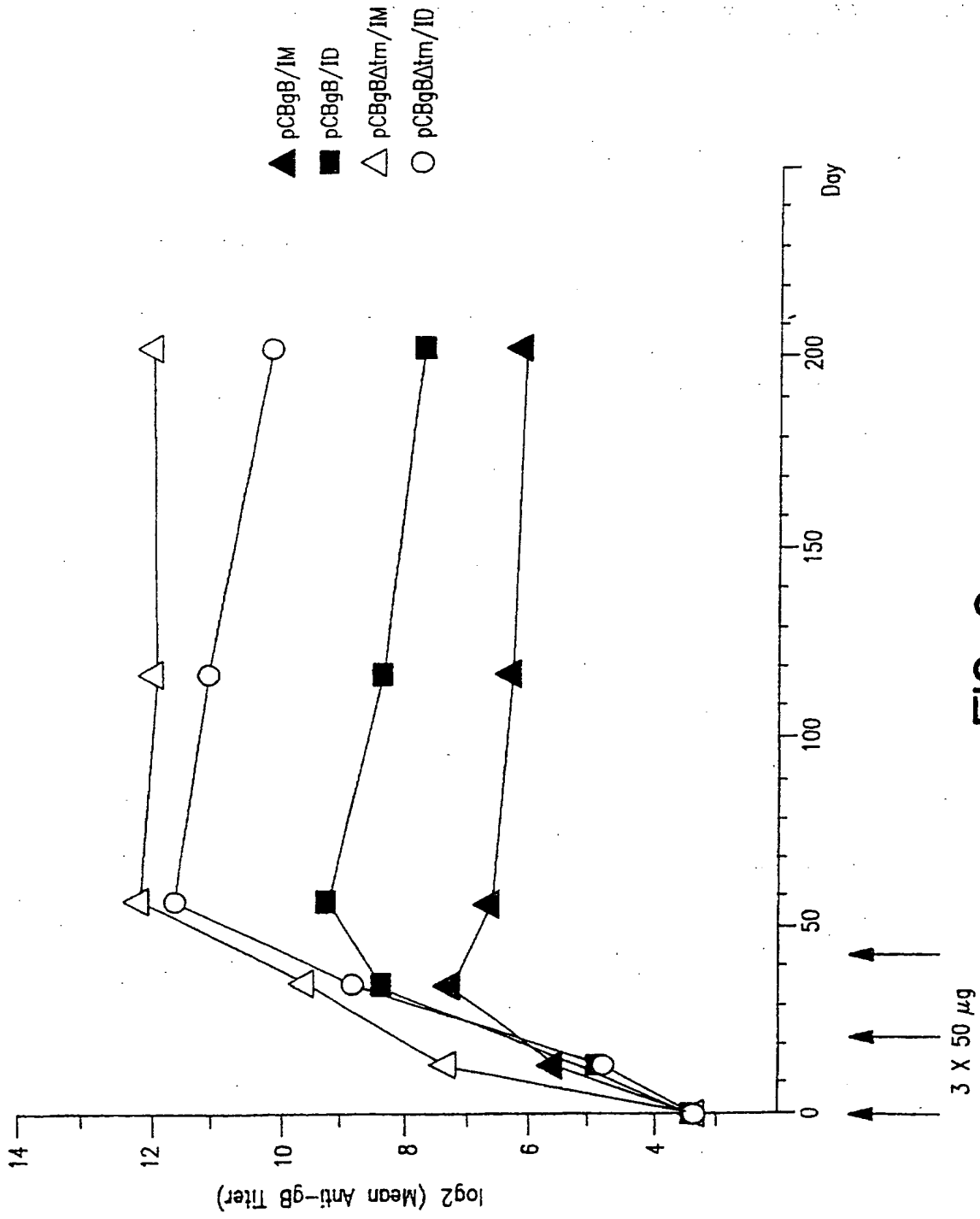

FIG. 9

INTERNATIONAL SEARCH REPORT

International A :ation No
PCT/US 97/06866

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/38 C07K14/045 A61K39/245 C12N15/86 //A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GÖNCZÖL, E. ET AL.: "Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate" VACCINE., vol. 13, 1995, GUILDFORD GB, pages 1080-1085, XP004057496	1,3,12, 16,17, 20-23, 26,27
Y	see the whole document	22,23, 26-28
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 1997	01.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Andres, S

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/06866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BERENCSI, K. ET AL.: "The N-terminal 303 amino acids of the human cytomegalovirus envelope glycoprotein B (UL55) and the exon 4 region of the major immediate early protein 1 (UL123) induce a cytotoxic T-cell response" VACCINE., vol. 14, April 1996, GUILDFORD GB, pages 369-374, XP004057290 cited in the application see the whole document	22,23, 26-28
X	EP 0 609 580 A (CHIRON CORP) 10 August 1994 see page 8, column 1 - column 45 see examples	1-3,12, 16,17, 21-23, 26,27
O,X	GONCZOL, E. ET AL.: "Preclinical evaluation of an ALVAC (canarypox)-human cytomegalovirus glycoprotein B vaccine candidate;immune response elicited in a prime/boost protocol with the glycoprotein B subunit." SCANDINAVIAN JOURNAL OF INFECTIOUS DISEASES, SUPPLEMENT 99, 1995, (110-112)., XP002041029 see the whole document	1,3,12, 16,17, 21-23, 26,27
A	DHAWAN, J. ET AL.: "Tetracycline-regulated gene expression following direct gene transfer into mouse skeletal muscle" SOMATIC CELL AND MOLECULAR GENETICS, vol. 21, 1995, pages 233-240, XP002041030 cited in the application	
A	BERENCSI, K. ET AL.: "MURINE CYTOTOXIC T CELL RESPONSE SPECIFIC FOR HUMAN CYTOMEGALOVIRUS GLYCOPROTEIN B (GB) INDUCED BY ADENOVIRUS AND VACCINIA VIRUS RECOMBINANTS EXPRESSING GB" JOURNAL OF GENERAL VIROLOGY, vol. 74, 1993, pages 2507-2512, XP002026070 cited in the application	

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/06866

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PANDE, H. ET AL.: "HUMAN CYTOMEGALOVIRUS STRAIN TOWNE PP65 GENE: NUCLEOTIDE SEQUENCE AND EXPRESSION IN ESCHERICHIA COLI" VIROLOGY, vol. 182, no. 1, May 1991, pages 220-228, XP000561310 ---	
A	GOSSEN M ET AL: "TIGHT CONTROL OF GENE EXPRESSION IN MAMMALIAN CELLS BY TETRACYCLINE-RESPONSIVE PROMOTERS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 89, no. 12, 15 June 1992, pages 5547-5551, XP000564458 ---	
A	EP 0 252 531 A (BEHRINGWERKE AG) 13 January 1988 -----	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internatic application No.
PCT/US 97/06866

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 17-28 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International A :ation No
PCT/US 97/06866

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0609580 A	10-08-94	AU 641121 B	16-09-93
		AU 3041389 A	25-08-89
		DK 179290 A	28-09-90
		EP 0436537 A	17-07-91
		JP 2607712 B	07-05-97
		JP 3503478 T	08-08-91
		WO 8907143 A	10-08-89
		US 5547834 A	20-08-96
EP 0252531 A	13-01-88	AU 605155 B	10-01-91
		AU 7412887 A	17-12-87
		DE 3644924 A	14-04-88
		DE 3782867 A	14-01-93
		ES 2044881 T	16-01-94
		JP 62296893 A	24-12-87